Universidad Autónoma de Madrid
Departamento de Biología Molecular
Facultad de Ciencias

The role of cell competition in tumour initiation and progression in *Drosophila melanogaster*

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Luna Laura Ballesteros Arias

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Luna Laura Ballesteros Arias – Licenciada en Biología

Director de la tesis:
Prof. Ginés Morata Pérez

Tutor de la tesis:
Prof. Ernesto Sánchez-Herrero

Madrid

A Gema

Resumen

Durante el desarrollo de un organismo multicelular pueden aparecer mutaciones espontáneas o debidas a estrés ambiental. Si estas mutaciones aportan una ventaja proliferativa o de supervivencia podrían inducir la aparición de tumores. Teniendo en cuenta la gran cantidad de divisiones celulares que tienen lugar para el desarrollo de una persona, la tasa de mutación y el elevado número de oncogenes y genes supresores de tumores, es probable que la aparición de células tumorales sea frecuente. La aparición de tumores, sin embargo, no lo es.

En *Drosophila* se describió el fenómeno de la *competición celular*, que garantiza la calidad de los tejidos. Su función principal es la identificación y eliminación de células aberrantes que puedan aparecer durante el desarrollo. Células con potencial tumoral también son detectadas por competición celular, lo que indica que este fenómeno puede tener un importante papel en la eliminación de tumores antes de ser clínicamente detectables.

Hemos analizado el papel de la competición celular en el desarrollo de tumores en *Drosophila*. Demostramos que es capaz de eliminar células con la ruta endocítica alterada y gran potencial tumoral. Además, mostramos cómo un grupo suficientemente grande de células tumorales puede formar un microambiente protector que permite el crecimiento del tumor.

En este trabajo describimos como la competición celular en la zona de interacción de las células tumorales y no tumorales puede promover el crecimiento del tumor tras la formación del microambiente. La competición celular genera una señalización mitogénica de las células apoptóticas que se traduce en una excesiva proliferación. En estas condiciones demostramos que células no tumorales son reclutadas al tumor, contribuyendo a su crecimiento.

Hemos utilizado otro modelo tumoral para analizar la señalización mitogénica de las células apoptóticas. Por ahora hemos estudiado el papel de una señal, Wingless (Wg), y el mecanismo por el cual induce proliferación extra en células vecinas. Observamos que Wg debe difundir para inducir sobrecrecimientos en nuestro modelo tumoral. Esto lo consigue por la estimulación de la proliferación y por la generación de focos nuevos de células que expresen *wg*. Éste mecanismo podría explicar los sobrecrecimientos asociados a competición celular en otros modelos tumorales. Nuestros resultados sobre la señalización proliferativa de células apoptóticas indican que algunos tratamientos antitumorales pueden ser contraproducentes.

Por último, hemos buscado nuevos elementos o funciones celulares que podrían mediar la identificación de células tumorales por competición celular.

Summary

During the life of a multicellular organism mutations may arise spontaneously or due to environmental stress. The acquisition of a survival or a proliferative advantage by mutant cells could drive the appearance of tumours. These events are likely to be frequent in humans, taking into account the great number of cell divisions that take place, the mutation rate and the large amount of oncogenes and tumour suppressor genes. However, during the life of an individual, tumours rarely appear.

A phenomenon was described in *Drosophila* that supervises the quality of tissues, termed *cell competition*. Its principal function is the identification and subsequent elimination of aberrant cells that may appear during development. This surveillance function includes the detection of potentially oncogenic cells. Thus cell competition may play an important role in eliminating tumours before they are clinically detectable.

We have analysed the role of cell competition in *Drosophila* tumorigenesis. We show that cell competition eliminates oncogenic cells in which the endocytic machinery is impaired and with high tumorigenic potential. In addition, we find that if oncogenic cells form a sufficiently large group, they create a protective microenvironment that allows the growth of the tumour.

We describe how cell competition occurring at the confrontation of tumour- and non-tumour cells can promote the growth of the tumour once a protective microenvironment has been created. Interestingly, cell competition causes mitogenic signalling from apoptotic cells that in turn results in overproliferation. We show that under these circumstances non-tumour cells are recruited to the tumour thus stimulating further growth.

We have also used a different tumour model to analyse the mitogenic signalling from apoptotic cells. We have so far tested the role of a particular signal, Wingless, and the mechanism by which it can induce extra proliferation of adjacent cells. We propose that Wingless spread is needed to induce overgrowths when there is a continuous signalling from apoptotic cells both through the stimulation of proliferation and through the generation of novel foci of *wingless*-expressing cells. This phenomenon could account for the overgrowths associated to cell competition in our tumour model. Our finding that apoptotic cells secrete proliferative signals responsible for tumour growth suggests that some of the current anti-tumour treatments administered to human patients may be counterproductive.

Finally we have performed a screen to identify candidate cellular functions or elements that could be involved in the specific detection of tumour cells by cell competition.

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Abbreviatures

A	Anterior compartment
act	*actin* promoter
ap	*apterous*
arm	*armadillo*
BCIP	5-bromo-4-chloro-3'-indolyphosphate
BDSC	Bloomington Drosophila Stock Center
BrdU	5-bromo-2'-deoxyuridine or bromodeoxyuridine
brk	*brinker*
BSA	Bovine Serum Albumin
Bst	*Belly spot and tail*
Casp3	*Caspase3*
D	Dorsal compartment
Diap1	*Death-associated inhibitor of apoptosis 1*
dlg	*discs large*
DOC	Deoxycholic acid
dpp	*decapentaplegic*
drice	Drosophila *interleukin converting enzyme*
dronc	*Death regulator Nedd2-like caspase*
en	*engrailed*
ESCRT	*Endosomal Sorting Complex Required for Transport*
Flp	*Flippase*
FRT	Flippase Recognition Target
fwe	*flower*
$Gal80^{TS}$	Gal80 temperature sensitive
H2	*Histone2*
hh	*hedgehog*
hid	*head involution defective*
hs	*heat shock* promoter
HS	Hybridization Solution
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JAK/STAT	Janus Kinase/Signal Transducer and Activator of Transcription
JNK	Jun-N terminal Kinase
LB	Luria-Bertani
lgl	*lethal giant larvae*
M	*Minute*
MARCM	Mosaic Analysis with a Repressible Cell Marker
mats	*mob as tumour suppressor*
Mmp1	*Matrix metalloproteinase 1*
NBT	Nitro-blue tetrazolium
NRT-wg	*neurotactin-wingless*
nub	*nubbin*
P	Posterior compartment
PBS	Phosphate buffered saline solution
PH3	*Phospho-histone3*

pnr	*pannier*
ptc	*patched*
puc	*puckered*
rab5^{KD}	*UAS dsRNA rab5* (ID: 34096, Bloomington Drosophila Stock Center)
rpr	*reaper*
sal	*spalt*
sav	*salvador*
SB	Staining buffer
scrib	*scribble*
TOPRO	To-Pro-3
TR-Dextran	Texas Red-Dextran
tub	*tubulin* promoter
UAS	*Upstream Activation Sequence*
ubi	*ubiquitin* promoter
V	Ventral compartment
w	*white*
wg	*wingless*
y	*yellow*
yap	*yes-associated protein*
yki	*yorkie*

INTRODUCTION

Cancer

The development of a multicellular organism of correct shape, pattern and size from a unique undifferentiated cell requires a fully orchestrated programme in which cell proliferation, cell death, cell differentiation and morphogenesis must be closely regulated. Digressions from the canonical developmental programme lead to defects and diseases that may compromise the adult functions or viability.

Cancer is an example of how an imbalance of cell proliferation and cell death may produce abnormal growth of parts of the body that can be life threatening. It is the second cause of human disease-driven death, accounting for about 14% of the total demises worldwide (*WHO report on non-communicable diseases*, 2014). It is defined as a complex group of genetic diseases of environmental or inherited origin that result in an uncontrolled overgrowth of a group of cells. A vast effort has been made by several platforms to detect recurrent genetic disorders and biomarkers in human tumour samples by making use of powerful sequencing and genomic technologies (reviewed in (Nakagawa et al., 2015)). This has revealed that most of human cancers share a few altered cell functions that govern the transformation of normal cells into tumourous ones. Also, there is clinical evidence of morphological lesions that indicate an evolution of the tissue from normal to malignant through a set of intermediate and reproducible stages (Eustis, 1989).

Taken together, it was proposed that tumour development responds to a multistep process incorporating several genetic alterations (Kinzler and Vogelstein, 1996; Thompson et al., 1989). The cellular traits that, when altered, drive the evolution of a tissue into a malignant cancer, were grouped into ten hallmarks: evasion from apoptosis, sustained proliferative signalling, evasion from growth suppression cues, cell motility, acquisition of immortality, induction of angiogenesis, evasion of immune detection, inflammation, genome instability and abnormal

regulation of cell metabolism (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). It is widely accepted that the acquisition of these characteristics occurs stepwise and without any particular order; they confer the cell a growth or survival advantage, as compared to the surrounding tissue, which induces a positive Darwinian selection of its lineage, and, consequently, the evolution of the tumour stage.

Types of cancer

In oncological research and medicine there is a need to classify tumours by grouping together cancer diseases that share similar characteristics, therefore permitting the transference of empiric knowledge among cases that share a taxonomical position in the classification. Different categories of cancer have been established depending on the trait of interest:

Affected tissue: the characteristics of the cancer cells, as well as the development and behaviour of the tumour, are different depending on the tissue where the cancer initially sets in. A first classification would distinguish between solid and circulating tumours. The former represent a class of overgrowing tissues that form a mass in the body, while the latter affect to hematopoietic cells. A second classification attends to the specific tissue in which the tumour arises. Cancer is named after its origin, being carcinoma (epithelial cells), sarcoma (soft tissues), leukemia (blood cells), lymphoma (lymphocytes), spinal cord and brain tumours, etc.

Among these subtypes of cancer the most prevalent in the population is the so-called carcinoma, which consists of many types of epithelial-origin cancers (breast, colon, lung, prostate, skin, etc.). In this work we present a study on tumours of epithelial origin.

Histopathological classification: early work on cancer was based on light- or electron-microscopy observation of dissected tissues from patients or animal models. Hence, they described morphological characteristics of the available tissues without a timeline analysis of its progression. A classification was created following histopathological observations (Eustis, 1989): 1) hyperplasia is an increase in the number of normal cells in an organized tissue; 2) dysplasia responds to an increased proliferation of cells that present some alteration, such as a failure to reach final differentiation; 3) benign neoplasias develop from a hyperplastic scenario. They are defined as dysplastic overgrowths that bulge out of the tissue but present a well-demarcated border and a uniform pattern; 4) malignant neoplasias are undefined masses of heterogeneous cell population in terms of differentiation status. They are able to metastasize although this characteristic is not needed to be considered malignant neoplasias.

Molecular and genetic classification: as indicated above, there are several platforms accumulating a massive load of molecular data from human samples of different tumours (The Cancer Genome Atlas or TCGA, The International Cancer Genome Consortium or ICGC). They take advantage of the variety of available techniques to perform whole genome sequencing, high throughput arrays, transcriptomics, etc. Importantly, they have developed powerful unsupervised protocols to analyse the outcome of such immense databases. As a result, they are defining the molecular and genetic traits of each particular cancer sample and grouping those that share similar genetic characteristics. They are providing a strong basis to develop specific treatments targeting the molecular alteration with higher life expectancy and lower side effects. Also, and very importantly, they are demonstrating that these criteria result in a more comprehensive and accurate clustering of tumours than the histopathological observations or the tissue specificity (few examples of these studies are (Brat et al., 2015) or www.icgc.org).

Cancer model organisms

Molecular analysis has revolutionized cancer research. Extensive work is being done to describe mutations and molecular pathways implicated in tumour formation in model organisms. This has been helpful to understand the datasets obtained by massive genomic analysis of cancer samples. Interestingly, it has been established that there are molecular pathways that not only gave rise to cancer when misregulated in model organisms, but also appear to be altered in human samples. Conversely, new emerging pathways involved in human tumours can be studied and characterized in model organisms. This transfer of knowledge between the study of human pathologies and research in model organisms is possible because of the conservation of cellular processes, biochemical machinery and gene function throughout the evolution of multicellular organisms. The advantage of studying basic processes in model organisms is that there are many experimental approaches that cannot be used in investigating human cancers.

Probably the experimental organism most suitable to be compared to humans is the mouse. It is a vertebrate model that shares about 95% of its genome with humans and therefore genetic diseases are highly comparable. There are mouse models for most of the human diseases and humanized mice that express human alleles and reproduce their associated phenotypes. Also it is well-stablished as a laboratory model due to the possibility to be genetically manipulated and its reasonably short life cycle (2-3 years) (reviewed in (Frese and Tuveson, 2007)).

Drosophila melanogaster

Although not so closely related to humans (61% of genome similarity), the fruit fly *Drosophila melanogaster* is also being used to study basic processes in human development and disease. Both organisms share key molecular pathways controlling patterning during development, cell proliferation, cell death or organogenesis. Since the release of its genome sequence in 2000 (Adams et al., 2000), the importance of *Drosophila* as a model to study human diseases and processes has substantially increased, mainly due to the fact that 77% of the human disease-related genes are conserved in flies and have comparable functions (Reiter et al., 2001).

A century of genetic manipulation in *Drosophila* has resulted in a powerful genetic toolkit to study biological processes. A constellation of sophisticated techniques allows a very precise manipulation of gene expression and function to investigate any gene of interest in time and space.

Drosophila offers other advantages for researchers: it is a small animal (the adult fly measures about 3mm) and needs a small amount of space to be stored and grown. It is cheap to maintain and obtain as there are public collections of genetically modified flies that are shared worldwide. It feeds naturally on rotting fruit, and fly food recipes have been developed in the laboratory that permit its maintenance. It is easy to breed and each female can lay up to 60eggs/day during its maturity. Generation time of *Drosophila* is about 10 days at 25°C, a short time when compared to other organisms, and which speeds up research. It possesses only four pairs of chromosomes, what makes tracking of mutations easier, aided by the existence of balancer chromosomes (which contain inversions that suppress homologous recombination during meiosis and carry dominant mutations to recognise their presence through adult or larvae phenotypes). Moreover, there is very little genetic redundancy in *Drosophila*, what facilitates the genetic analysis of the biological processes under study.

Brief introduction to *Drosophila* development

Drosophila melanogaster is a holometabolous insect, that is, it undergoes a complete metamorphosis. The embryonic period lasts 24h at 25°C. In the first three hours of development, several nuclear divisions take place in a syncytial blastoderm, followed by cellularization. Before egg hatching there are morphogenetic movements that form the endo-, meso- and ectodermic layers of the embryo. The embryonic period is followed by three larval stages that take four days at 25°C. Most of the growth occurs during this period. The larva harbours a set of epithelial sacs called imaginal discs that are the precursors of the adult

cuticular structures. They are named after the adult structure they form, *i.e.* wing disc, leg disc, eye-antennal disc and so forth.

At the end of the larval stage, the organism stops feeding and encapsulates in a puparium where complete metamorphosis takes place. This takes 4-5 days at 25°C. During this period all the imaginal discs differentiate their corresponding adult structures.

Imaginal discs – the wing disc

At the end of embryogenesis, discrete groups of ectodermal cells invaginate and become the primordia of the imaginal discs, which will grow during the larval stages. Imaginal discs (figure I1) are sacs of cells organized as a monolayer of a pseudostratified simple epithelium that confront the apical region leaving the lumen in between. Due to their very simple structure and the ease of their manipulation, they have become a very convenient model to analyse epithelial dynamics.

We have used the wing imaginal disc as our model to study epithelial tumour progression. It has been predicted that the primordium of this disc consists of 30-55 cells by the beginning of the first larval stage. During early larval stages it undergoes rapid cell divisions that slow down during the late third larval period, reaching a final size of about 30,000 cells at pupariation (M. and Schneiderman, 1977; Martin et al., 2009). During metamorphosis the wing disc evaginates so that the central-most region of the disc (known as the wing pouch) creates the adult wing blade while the surrounding tissue makes the wing hinge and the adult thoracic body wall (figure I1A).

Upon the invagination of the imaginal discs primordia, a subset of these cells expresses the selector gene *engrailed* (*en*), which identifies them as posterior (P) cells (Morata and Lawrence, 1975). The lack of *en* identifies the remaining cells as anterior (A) (figure I1B). Importantly, cells descending from an *en*-expressing cell will share this identity, thus forming an independent lineage unit. The expression of *en* confers the cells with specific adhesion properties that prevent them from mixing with those of anterior origin. These independent units are called compartments, and are the basic components of the *Drosophila* body plan (Garcia-Bellido et al., 1976; Steiner, 1976).

In the wing imaginal disc there is a later compartment subdivision established by the expression of another selector gene: *apterous* (*ap*). *ap* is expressed in a group of cells of both the A and P compartments and defines the dorsal (D) compartment (figure I1B). Cells lacking *ap* define the ventral (V) compartment (Blair, 1994; Díaz-Benjumea, 1993).

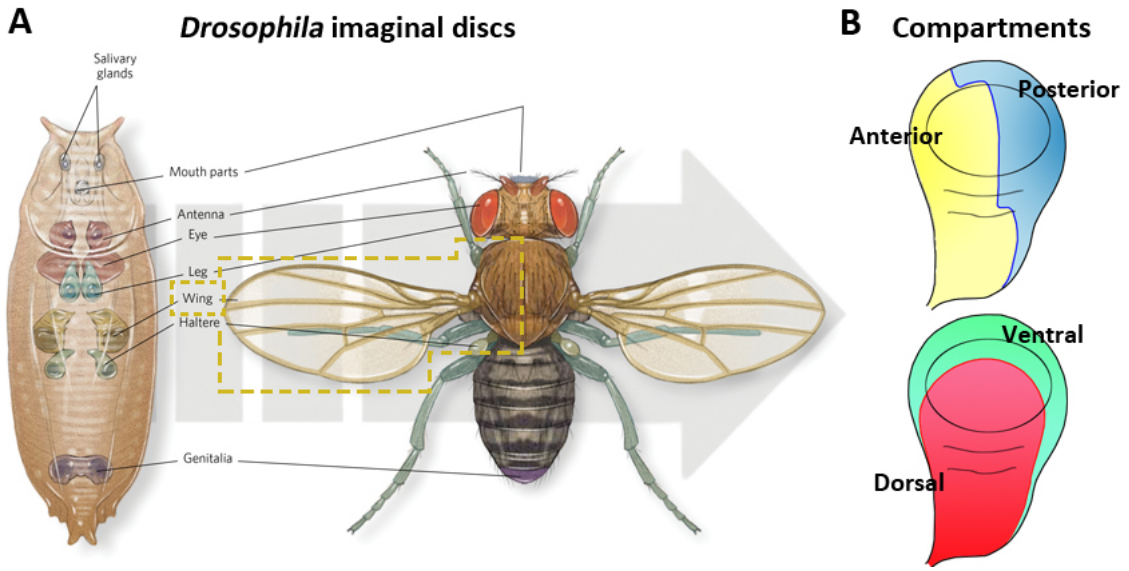


Figure I1. *Drosophila* imaginal discs. Imaginal discs grow during larval development. During metamorphosis they differentiate the adult cuticular structures **A**. We outline (yellow dashed line) the structures that differentiate one wing imaginal disc: one wing and one heminotum. Wing discs are divided during development into lineage-restricted compartments: there is a first subdivision into Anterior and Posterior compartments and later into Dorsal and Ventral (**B**). Image modified from (Grewal, 2013). Originally designed by Catherine Delphia.

The confrontation of two compartments is essential because the boundary becomes an organizing centre of the disc, playing a role in disc growth and generating the wing pattern. In the P compartment *en* triggers the expression of the gene *hedgehog* (*hh*) coding for a protein that acts as a morphogen. Hh function is blocked in P by the activity of En. However, it spreads anteriorly and induces the expression of *decapentaplegic* (*dpp*) in the most anterior stripe of cells (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Dpp functions also as a morphogen and disperses creating a long-range concentration gradient from the A/P border to the edges of the disc (Zecca et al., 1995). Cells respond to Dpp by activating different target genes of its pathway in a concentration-dependent manner (Kim et al., 1996; Lecuit et al., 1996; Nellen et al., 1996). Thus, the Dpp gradient induces a gene-expression pattern that will turn into the correct specification of the cells and the function of the organ, in this case, the wing. Similarly, the confrontation of the D and V compartments induces the activation of Notch in the cells of both D and V cells adjacent to the compartment boundaries. This activity of Notch induces the expression of *wingless* (*wg*)(Kim et al., 1995). Wg acts as a morphogen spreading from its source cells and activating its target genes in a concentration-dependent manner (Neumann

and Cohen, 1997; Zecca et al., 1996). Overall, cells in the wing disc receive spatial information that leads to a fine-tuned pattern of structures and identities within the organ.

The role of Wg and Dpp in the induction of proliferation is somewhat controversial. There is enough evidence to think that they regulate tissue proliferation although their direct role is still under revision. Functional experiments have shown that the ectopic activity of Wingless can induce extra proliferation of adjacent cells (Diaz-Benjumea, 1995; Giraldez and Cohen, 2003; Neumann and Cohen, 1997). The vertebrate Wnt pathway, ortholog of the Wg pathway in *Drosophila*, has also been linked to growth stimulus in several models of epithelial tumours or regenerating buds (reviewed in (Clevers and Nusse, 2012))(Chera et al., 2009). However, the response to this morphogen in the wing disc seems to depend on the region where it is active, as in cells flanking the D/V boundary the activity of Wg is crucial to block proliferation and therefore control the final size of the organ (Johnston and Sanders, 2003). Recent work also questions the role of Wg in the induction of proliferation at a distance. In these experiments, a fusion of *wg* and a sequence of *neurotactin* (*nrt*), *NRT-wg*, whose product is tethered to the cell membrane and therefore does not spread thrpugh the disc, substitutes the normal *wg* gene. Flies containing Wg-NRT as the sole source of Wg function survive and are of near normal size (Alexandre et al., 2014). The role *dpp* plays in growth promotion is also a major issue of debate nowadays. While it has been proven that ectopic expression of *dpp* drives local proliferation and duplications (Capdevila and Guerrero, 1994; Martin-Castellanos and Edgar, 2002; Zecca et al., 1995), it seems to play its function through repression of another gene, *brinker* (*brk*) (Martin et al., 2004; Muller et al., 2003).

Cancer in *Drosophila melanogaster*

When we planned to study cancer using *Drosophila* as a model organism, the first question that came to our minds was: “but does *Drosophila* develop cancers without genetic manipulation?”. And the answer is “Yes, it does”. Although it has a short adult lifespan when compared to mammalian organisms (about 40 days at 25°C), it ages and its cells accumulate mutations so that the probability of acquiring a malignant transformation increases. Indeed, it was reported that ageing was correlated with the appearance of gut and testis spontaneous tumours in *Drosophila* (Salomon and Jackson, 2008).

It has been proposed that about 75% of tumour growth occurs before being clinically detectable and before acquiring a metastatic behaviour (Campbell et al., 2010; Moreno, 2008; Yachida et al., 2010). This reveals the great importance of analysing the first stages of tumour

development. *Drosophila* has become a convenient model to study several aspects of cancer initiation and progression. There are genetic tools that allow us control the exact time when tumour cells are generated. Therefore, we can examine the events from the time a single cell becomes tumorous until a possible metastasis occurs.

Drosophila can serve as a model in two different ways. First, many human cancers have been associated with specific mutations or altered pathways and homologous genes to those mutated are often present in *Drosophila* (reviewed in (Rudrapatna et al., 2011) and (Gonzalez, 2013)). The oncogenic potential of these alterations can be studied experimentally in this model.

Second, analysis of *Drosophila* mutants led to the discovery of conserved tumour suppressor genes and oncogenes whose human homologs are also associated with development of tumours. Here I briefly describe several *Drosophila* pathways known to be involved, when altered, in tumorigenesis.

Apico-basal polarity

There are several protein complexes in *Drosophila* that act together to create a cell apico-basal polarity axis and generate cell-cell junctions in epithelial cells. These modules are the Scribble, the Par and the Crumbs complexes (reviewed in (Assemat et al., 2008) and (Tepass et al., 2001)). Misregulation of their components leads to altered cell polarity and a loss of epithelial structure. Interestingly, these altered cells also lose their ability to stop growth at the proper time hence inducing overgrowth (reviewed in (Khursheed and Bashyam, 2014)). It has been observed that mutant larvae for *lethal giant larvae* (*lgl*), a component of the Scribbled complex, develop neoplastic overgrowths of their imaginal discs and central nervous system. These animals extend their larval period and die as gigantic larvae (hence the name of the gene) unable to pupate (Gateff, 1978; Menendez et al., 2010). Cells mutant for *lgl* grow at approximately the same pace as wild type cells do, but continue dividing as long as the larva is alive (Menendez et al., 2010). Mutations in other components of the apico-basal polarity complexes show comparable behaviour (reviewed in (Humbert et al., 2008), which suggests that they may be participating of a common pathway controlling cell proliferation (Bilder et al., 2000).

Endocytic pathway

The distribution of membrane-associated proteins is not homogeneous in polarized epithelial cells. To maintain it cells can endocytose and relocate these proteins or degrade them if they

are not further required. There are several endocytic pathways in *Drosophila*. In the canonical pathway (figure I2) the plasma membrane invaginates so that the cargo is retained within the vesicle (extracellular cargo) or at the membrane of the vesicle (membrane-bound cargo). After the vesicle detaches from the cell membrane, it fuses with other vesicles creating the early endosome from which the cargo can either be recycled to the membrane or degraded by fusion of endosomes with lysosomes.

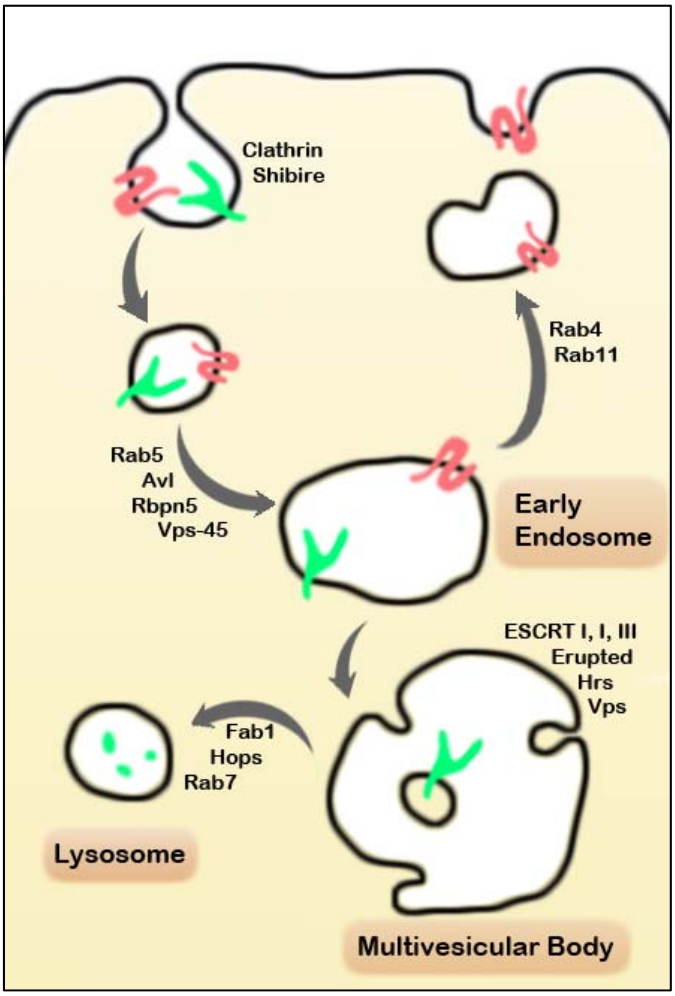


Figure I2. Clathrin-dependent endocytic pathway in *Drosophila*. We have depicted here the main structures formed during vesicle trafficking and some of the proteins involved in each step. The green cargo is endocytosed and transported for degradation in the lysosome. Instead, the pink cargo is endocytosed and recycled back to the cell surface membrane.

Several protein complexes regulate and control this mechanism, some of them being: AP-2/Clathrin/Shibire, involved in the membrane invagination and scission, Rab5/Avalanche/SNARE, which deliver the vesicle to the early endosome, Rab4/Rab11, that belong to the recycling machinery, and Hrs/ESCRT, which belong to the degradation route (reviewed in (Shivas et al., 2010)). Interestingly, many components of the endocytic machinery have also been linked to growth control as mutations of their encoding genes give rise to neoplastic tumours in imaginal discs and central nervous system (Lu and Bilder, 2005; Moberg

et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). Hypomorphic mutant larvae for these genes have a prolonged larval period and die during pupal stage. Several hypotheses have been raised to explain how the endosomal trafficking could suppress tumour growth. It could compromise the degradation of Crumbs, an apical protein that, when overrepresented in a tissue, triggers a tumorous transformation (Lu and Bilder, 2005; Moberg et al., 2005). Another possibility is that the blockage of endocytosis induces the accumulation of activated molecules (such as Notch) that could be signalling in the cytoplasm for a prolonged time (Vaccari and Bilder, 2005).

Hippo pathway

Cells have the ability to transduce external cues into transcriptional responses and establish dynamic interactions with their environment. One such mechanism is the Hippo pathway, which integrates signals related to cell-cell contact, tension, cell polarity or positional information given by morphogens into regulation of cellular metabolism, survival or proliferation. The Hippo pathway is the principal regulator of cell proliferation in *Drosophila*. It is conserved in vertebrates (Dong et al., 2007; Huang et al., 2005), where it performs a similar function.

Its regulation is nowadays a complex and active field of research, although it is accepted that its core components are a hierarchical cascade of kinases that, through a series of phosphorylations regulate the subcellular localization of the transcriptional co-factor Yorkie (Yki). Most of its regulation relies on Hippo, which, when activated, proceeds to the phosphorylation of Warts, Mob as tumour suppressor (Mats) and Salvador (Sav). The kinase Warts phosphorylates three residues of Yki triggering its interaction with 14-3-3 proteins, which retain Yki in the cytoplasm, avoiding its translocation to the nucleus. When the pathway is inactivated, Yki enters the nucleus. Nuclear Yki associates to site-specific co-factors and promotes the transcription of genes involved in regulation of cell growth, like the *bantam microRNA* or *dMyc*, of cell cycle progression, like *E2F1* and *cyclins A, B* and *E,* or inhibitors of apoptosis like *Diap1*. Moreover, it transcribes activators of the pathway thus creating a negative feedback loop that ends up recruiting Yki to the cytoplasm (reviewed in (Irvine, 2012) and (Staley and Irvine, 2011)). Although these components of the pathway are conserved in sequence and function in vertebrates, the upstream regulation of the cascade seems to have variations between these *phyla* (reviewed in (Halder and Johnson, 2010)).

The cell proliferation program that this pathway regulates already suggests a possible role in tumorigenesis, and indeed its misregulation has been linked to several types of cancer. Loss-of-

function mutations of the core elements of the cascade (*hippo*, *wts*, *mats* or *sav*) leads to translocation of Yki into the nucleus and induction of cell proliferation and survival, followed by hyperplastic overgrowth of the tissue (Harvey et al., 2003; Justice et al., 1995; Lai et al., 2005; Tapon et al., 2002; Wu et al., 2003). Human orthologs have been also linked to organ size control. An example is the knocking-down of the pathway in the liver or the hyperactivation of the *yki* ortholog *yap*, which results in a hepatocellular hyperplastic carcinoma (Dong et al., 2007; Lu et al., 2010).

Misregulation of apoptosis – undead cells

Programmed cell death (apoptosis) is a highly conserved process aimed to eliminate damaged or superfluous cells. Several apoptotic mechanisms have been described, with important genetic, cellular, and physiological differences among them (Fuchs and Steller, 2015). As I have mentioned above, one of the hallmarks of cancer is the ability of tumour cells to evade apoptosis (Hanahan and Weinberg, 2000). Recent advances in the apoptosis field, especially in *Drosophila*, have provided significant cues about the role apoptosis plays in tumorigenesis.

Apoptotic program in *Drosophila*

Experimental analysis of apoptosis was initially performed in *Caenorhabditis elegans* (Ellis and Horvitz, 1986) and since then it has been characterized in several other organisms. It is the major mechanism of cell removal upon stress or developmental cues. It consists of a series of steps that finally lead to the activation of effector caspases, proteases that are ubiquitously expressed as inactive zymogens. Cellular stress is sensed by two different elements in *Drosophila*, depending on the nature of the signal (DNA damage, reactive oxygen species, lack of nutrients…): the Jun-N terminal Kinase (JNK) pathway and the transcription factor *dp53* (figure I3). JNK (Tumour Necrosis Factor, TNF homolog) functions both in *Drosophila* and in vertebrates and has a wide range of effects when activated, having a prominent role as an apoptosis-inducer mechanism. Similarly, *p53* plays several roles. In vertebrates it is involved in cell-cycle arrest upon DNA replication errors or cellular senescence. However, it has been mainly linked to the induction of apoptosis in response to stress agents such as ionizing radiation. These effects have also been observed in *Drosophila* in experiments of overexpression of *dp53*, the ortholog of *p53* (reviewed in (Steller, 2000)).

It has been shown that *dp53* binds to, and activates, the transcription of the proapoptotic gene *reaper* (*rpr*) (Brodsky et al., 2000). Similarly, it has been suggested that *rpr* and *head involution defective* (*hid*), another proapoptotic gene, are transcriptionally regulated by the JNK transcription factors *jun* and *fos* (Fan et al., 2009; McEwen and Peifer, 2005). *rpr* acts together

with *hid* and *grim* to bind *Drosophila* Inhibitor of Apoptosis Proteins (dIAPs) and antagonize their activity. In *Drosophila* these genes block Diap1, which functions as an E3-ubiquitin ligase targeting for degradation the apical Caspase-9 (Dronc in *Drosophila*). Upon a death stimulus Diap1 promotes self-conjugation and therefore degradation allowing the evolution of apoptosis through Dronc activity. The apical caspase Dronc cleaves the effector caspases Dcp1, Drice (*Drosophila* interleukin converting enzyme) and Diap1 itself (reviewed in (Steller, 2008)). Dcp1 and Drice are cysteine proteases that cleave cellular proteins and bring about apoptosis (figure I3).

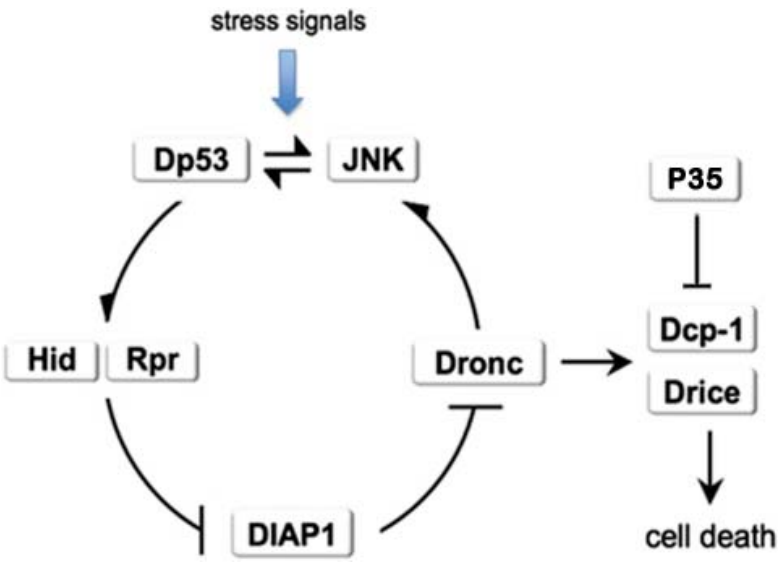


Figure I3. Apoptotic pathway in *Drosophila*. Here we show a simplified version of the elements involved in the apoptotic pathway in *Drosophila*. Cellular stress activates either *p53* or the JNK pathway, depending on the nature of the signal. These transcriptionally activate *hid* or *rpr*. Their products stimulate the degradation of the Diap, which actively blocks the apoptosome in which Dronc participates. The apoptosome cleaves and activates the effector caspases, which drive the degradation of the cellular structures and thus cell death. The baculovirus protein P35 binds to and inactivates the effector caspases. Image modified from (Shlevkov and Morata, 2012).

It has been recently reported that not only JNK and *dp53* are upstream the apoptotic pathway but are also activated downstream of Dronc, therefore suggesting a feedback loop that ensures the execution of cell death once the apoptotic machinery has been activated (Shlevkov and Morata, 2012) (figure I3).

Signalling from apoptotic cells

Dying cells were long thought to have low capacity to signal to their environment due to their rapid clearance from the tissues. However, there is recent evidence that cells that enter the apoptotic program secrete apoptotic and proliferative signals before dying.

An example of this is the massive cell death that may happen during development to refine organ structures and that leads to a secondary wave of cell death in nearby cells. This has been observed both in *Drosophila* imaginal discs and in mammalian hair follicles, and is mediated by Eiger signalling from apoptotic cells (Perez-Garijo et al., 2013). Eiger is a soluble ligand that binds to its receptors Wengen and Grindelwald to activate JNK (Andersen et al., 2015; Kauppila et al., 2003) inducing apoptosis of receiving cells. This non-autonomous induction of apoptosis may play a role in the complete elimination of tissues that are not further required. It has been hypothesized that this phenomenon could be the explanation for the bystander effect observed in radiobiology, in which non-irradiated cells standing next to irradiated cells acquire the death phenotypes commonly associated to irradiation (reviewed in (Prise and O'Sullivan, 2009)).

Another example of signalling from apoptotic cells to their neighbours is the case of the so-called *undead cells*. The experiments that led to the discovery of these cells consisted of the activation of the apoptotic machinery through a death stimulus on a cell population while blocking the effector caspases to avoid the elimination of the cells. Several death stimuli have been used, like the expression of proapoptotic genes, the loss-of-function of antiapoptotic genes (*Diap1*) (Huh et al., 2004; Ryoo et al., 2004) or the induction of stress by prolonged high temperatures or X-radiation (activating *dp53* and/or the JNK pathway) (Perez-Garijo et al., 2004). In all cases the blockage of apoptosis was done by expressing the baculovirus protein P35, which functions as an effector caspase (Dcp1, Drice) inhibitor (Clem et al., 1991) (figure I3). These experiments allowed the authors to study apoptotic cells for an extended period and observe their behaviour. It was reported that undead cells actively secrete mitogenic molecules such as Dpp or Wg, two important morphogens in *Drosophila* development (Perez-Garijo et al., 2004; Ryoo et al., 2004). Interestingly, this persistent signalling, which depends on the apical caspase Dronc and on JNK activity (Perez-Garijo et al., 2009; Ryoo et al., 2004), induced proliferation of surrounding cells leading to hyperplastic overgrowths (a schematic representation is shown in figure R20, in the Results section).

In this section, we have briefly reviewed how apoptosis may play an active role in tumour growth. On the other hand, apoptotic activity within a tumour is being induced to treat cancer (radio- and chemo-therapy), which mostly helps shrinking the tumour burden due to the loss of cells. Nevertheless, the idea that apoptotic cells could actively signal to their environment to promote extra cell death or proliferation could be of great importance, and should be taken into account and further studied in clinical trials.

Cell competition

Considering the number of cells of higher animals, their number of genes and the mutation rate of somatic cells, it is clear that somatic tissues must always contain numerous mutant cells in which basic physiological parameters (cell size, cell division, positional cell identity, etc.) may be compromised. Other somatic mutations may generate aberrant or malignant cells that may affect tissue development or fitness. There is evidence for the existence of an intrinsic mechanism to recognise and eventually eliminate those abnormal cells (figure I4). This mechanism was termed *cell competition* and, although it was first characterized in *Drosophila*, it has been reported to occur in vertebrates as well.

The first description of this phenomenon came from the analysis of a dominant mutation in a *Drosophila Minute* (*M*) gene that reduces ribosomal biogenesis and therefore slows down translation and protein synthesis. While homozygous mutations (*M/M*) of these genes are cell lethal, heterozygous (*M/+*) adults are viable and fertile, although they suffer a developmental delay. Interestingly, clones of heterozygous cells (*M/+*) growing in a wild type wing disc were not underrepresented in the adult wing, as expected, but completely eliminated (Morata and Ripoll, 1975). The authors concluded that these cells failed at competing for nutrients or growth factors due to their reduced growth rate. Since then, numerous cell competition scenarios have been described. There are some salient features of cell competition:

1. - It drives the death of one of the juxtaposed cell types. The fact that *M/+* clones generated during the larval stages of *Drosophila* were not represented in adults indicated that they were being actively eliminated. Later reports that made use of techniques for direct visualization of clones in developing tissues found that indeed that was the case. *M/+* cells growing within a wild type tissue activated the JNK pathway that, in turn, led to apoptosis (Moreno et al., 2002). Cell death occurred after 24-48 hours of clone initiation and in cells in direct contact with wild type cells.

2. - It is a context-dependent mechanism. An important characteristic of cell competition is that it induces apoptosis of cells that would not be eliminated when growing in a homotypic environment (*i.e*. not in a mosaic tissue formed of different cells). The cells are viable but die when adjacent to wild type cells.

3. - It is a short-range phenomenon. From the previous attributes it has emerged that cell competition may require direct interaction between competing cells. However, there are few observations that question this conclusion. It was reported that soluble factors could mediate cell competition in an *in vitro* model of cultured cells (Senoo-Matsuda and Johnston, 2007),

downgrading the importance of cell interaction. Such *killing signal* was also proposed to exist in serum-deprived cultured cells. In this scenario, the killing signal would induce in receiving cells a response similar to that observed in out-competed cells (Portela et al.).

4. - It does not take place across compartment boundaries (Simpson and Morata, 1981). Assuming that cell competition depends on short-range cell interactions, it should not have local constraints in a continuous epithelial tissue like the imaginal discs of *Drosophila*. However, it has been recently reported that the higher Myosin II-driven tension present in these boundaries is responsible for a lack of cell mixing and a less efficient cell-cell communication, thus impeding cell competition (Levayer et al., 2015).

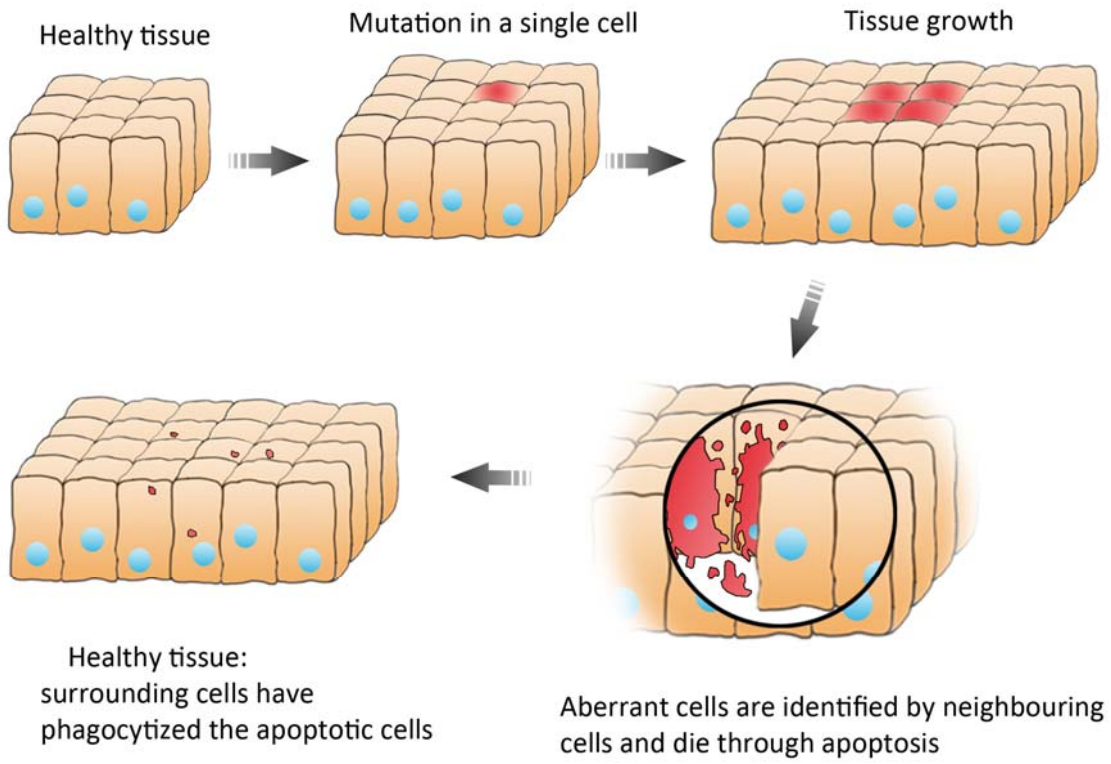


Figure I4. Cell competition. We exemplify the events following the alteration of an individual cell in a healthy epithelium based on our experience. Cell competition does not induce cell death of an unwanted cell type until 24-48h since its appearance, thus permitting a few cell divisions. After that time they are identified and die through apoptosis. Apoptotic cells tend to extrude basally although there is evidence that surrounding cells also phagocytize cell debris.

Role of cell competition

Tissue quality control

As mentioned above, spontaneous aberrant cells may appear in a growing tissue and compromise its function. Their selective elimination is then of great importance. This would be the case of *M/+* cells. Even though they would not represent a developmental problem on their own, their physiological status is somehow compared with that of their wild type neighbours and finally out-competed. An ortholog gene of *Drosophila Minutes* is the mouse *Belly spot and tail* (*Bst*) gene. Interestingly, it functions very much like *Minutes* in terms of cell competition: homozygous cells, although viable, are eliminated in a mosaic context (Oliver et al., 2004). Similarly, it has been reported that cells that do not acquire the right positional identity, are equally removed by cell competition (C. Estella, unpublished data and (Milan et al., 2002)).

Cell competition has also been observed at the confrontation of cells that bear different amounts of dMyc, which functions as a transcription factor that positively modulates cell metabolism and proliferation acting downstream of a number of growth-regulating pathways. Flies carrying a hypomorphic allele of dMyc are viable, while clones of this genotype growing in a wild type background are selectively eliminated by cell competition. The opposite experiment, in which the background cells express extra dMyc while the clone cells are wild type for dMyc, led to the concept of *supercompetition*. In this scenario cell competition detects wild type cells as unwanted and eliminates them (de la Cova et al., 2004; Moreno and Basler, 2004). This has been proposed to be a possible first step in cancer progression: a random cell would acquire, through a first mutation, a competitive advantage that would allow it to kill wild type surrounding cells and occupy a greater area. Consequent mutations could drive tumorigenic transformation (Casas-Tinto et al., 2011).

Very importantly, the role of cell competition has been reported to occur during normal development to select the best adapted cells to shape the organism (Claveria et al., 2013; Sancho et al., 2013). This was observed in the mouse epiblast, where the levels of dMyc vary among different cells, and those with lower dMyc, and hence metabolism, are eliminated, dMyc levels being consequently homogenized (Claveria et al., 2013). In this model it has also been proposed that diffusible signals may induce cell death upon co-culture (Sancho et al., 2013).

Other genetic contexts have been proved to induce cell competition against wild type cells. For instance, clones of cells with increased activity of the Janus Kinase/Signal Transducer and

Activator of Transcription (JAK/STAT) pathway grow at the expense of wild type surrounding cells (Rodrigues et al., 2012). Similarly, clones mutant for *warts* downregulate the Hippo pathway allowing the translocation of Yki to the nucleus, which in turn leads to overproliferation and cell competition against wild type neighbouring cells (Tyler et al., 2007). It was later reported that dMyc was a target gene of Yki and responsible for the wild type cell killing upon Hippo pathway downregulation (Ziosi et al., 2010). However, the implication of dMyc in the induction of supercompetition is yet to be clarified, as clones with high activity of the JAK/STAT pathway induce cell death of wild type cells independently of dMyc (Rodrigues et al., 2012).

In a study on cell repopulation it was observed that stem and progenitor cells injected in adult rat liver did repopulate the liver to a higher extent than expected. They suggest that cell competition is taking part of the process as these highly proliferating cells induce cell death of healthy resident cells of the liver to repopulate the liver (Oertel et al., 2006).

Altogether, these arguments suggest that cell competition acts by eliminating cells that, upon comparison with their neighbours, are less adapted to their context or have lower metabolic potential, thus improving the cell pool available for further development.

Tumour suppressing function of cell competition

From the definition of cell competition it can be hypothesized that cells that acquire oncogenic mutations may be also detected as different when compared to their neighbours inducing a cell competition response. This has been tested for various proto-oncogenes or tumour suppressor genes both in *Drosophila* and in *in vitro* models of mammalian cultured cells.

As previously presented, several tumour suppressor genes have been studied in *Drosophila*. By definition, their loss of function generates a tumoral transformation of the tissue (see "Cancer in *Drosophila*" section). However, the induction of mutant clones for those genes does not result in tissue overgrowths, as expected. Instead, they are eliminated after 24-48h of clone initiation by JNK-activated cell death. This has been reported for mutations of genes of the apico-basal polarity pathway, such as *lgl*, *scrib* or *dlg* (Brumby and Richardson, 2003; Grzeschik et al., 2010; Igaki et al., 2009; Menendez et al., 2010) (Tamori et al., 2010). Interestingly, *lgl*-mutant clones' elimination could be rescued by overexpressing *mahjong*, which codifies for a protein that interacts with Lgl in normal conditions. *mahjong* mutant clones are themselves eliminated in a wild type context, while mutant animals are phenotypically normal, which suggests that *lgl* elimination phenotype could be mediated by Mahjong (Tamori et al., 2010).

The characteristics of the elimination of the oncogenic clones closely resemble those of cell competition: first, they are context-dependent mechanisms (oncogenic cells are viable in an homotypic context but eliminated when growing in mosaic tissues with wild type cells); second, the timing of clone elimination corresponds to the timing of cell competition (Moreno et al., 2002) (these clones grow normally during the first 24h and after that they are detected); finally, there is a specific JNK activation that leads to apoptosis of the clone cells; apoptosis appears mostly at the clone borders.

Cell competition has also been recently reported to play a physiological role as tumour suppressor in a mammalian model. In the mouse thymus, there is a continuous replacement of resident T-lymphocyte precursors with younger incoming progenitors from the bone marrow. The newer precursors substitute for the residents by inducing apoptosis of the latter, what also fulfils the definition of cell competition. If cell competition is abolished, resident precursors hyperactivate an oncogene that at the end leads to a tumoral transformation into a T-cell acute lymphoblastic leukaemia (Martins et al., 2014).

It is therefore established that different potentially tumoral cells are recognised as unwanted and removed from the tissue by cell competition, which is therefore functioning as a tumour suppressor mechanism both in mammalian and *Drosophila in vivo* and *in vitro* models.

Mechanisms of cell competition

The importance of cell competition during normal and pathological development has increased the general interest on determining the molecular mechanism that underlies it. Much effort has been put recently to uncover pathways that function in cell competition models.

In a genomic screen designed to detect variations in gene expression in a cell competition context in *Drosophila*, several genes were found to be upregulated early after the induction of the competitive interaction, suggesting a possible implication at the initiation of the process (Rhiner et al., 2010). From this seminal study the authors have proposed a pathway to explain how cell competition may detect and eliminate cells. The confronting cell types appear to compare relative levels of the ubiquitously expressed isoform of the transmembrane protein Fwe, Fwe^{ubi}, which directs the elimination of the cell type with lower levels. Otherwise, out-competed cells can be tagged by the presence of the two other isoforms of Fwe, Fwe^{LoseA} and Fwe^{LoseB} (Rhiner et al., 2010). Once recognized as weaker cells, they autonomously trigger the expression of *sparc*, which provides a transient blockage of a yet unknown diffusible killing signal, understood as a safeguard mechanism to avoid the elimination of healthy cells (Portela

et al.). If the fitness comparison continues and the cell has to be eliminated, it does so through the activation of *azot*, meant to eliminate such abnormal cells during development (Merino et al.). This molecular mechanism was reported in several models of cell competition, including dMyc-driven supercompetition, *Minute* clones and *dpp*-based competition. However, in tumour models of cell competition it has not been proved yet, although it was reported that the $Fwe^{LoseA/B}$ isoforms are specifically expressed in *scribble* mutant clones (Rhiner et al., 2010).

More recently, a different mechanism was proposed to be in charge of the elimination of wild type cells surrounded by cells expressing high dMyc (dMyc supercompetition) and of *Minute* heterozygous cells in a wild type background. In this case, two known pathways are involved in cell detection and killing by cell competition: the Toll and the Imd pathways. The Toll pathway has a role during *Drosophila* embryo dorso-ventral axis establishment, and both pathways are components of the innate immune system of the organism. The activation of these pathways through the recognition of non-self or altered-self cells (bacteria, virus-infected or cancer cells) leads to the emission of anti-microbial peptides meant to block and eliminate them. It has been shown that the elements of these pathways are co-opted to recognize and induce the expression of the proapoptotic *hid* and *rpr* genes in unfit (and hence, killing) cells in cell competition models (Meyer et al., 2014). Again, information is lacking about the possible implication of this mechanism in the elimination of tumour cells through cell competition.

OBJECTIVES

This work has been carried out in a laboratory with long experience in *Drosophila* development and in the phenomenon of cell competition. Our scope in this thesis is to provide experimental evidence to understand the role that cell competition plays in tumour initiation and progression and the mechanisms involved. To achieve this general goal, we have addressed some more specific objectives, as follows:

1. To validate loss of *rab5* activity as a model to study cell competition.
2. To determine the conditions required to generate a protective microenvironment to overcome cell competition and initiate a tumour.
3. To study the role of cell competition-derived apoptosis in tumour progression.
4. To analyse the mechanism and role of Wingless in the induction of hyperplastic tumours.
5. To identify possible factors that mediate the onset of cell competition.

MATERIALS AND METHODS

Fly care

Flies were kept in a standard culture medium and in incubation chambers with controlled temperature and relative humidity (75%). Depending on the experiment, they were raised at 17°C or 29°C, as indicated in the text. If not indicated, they were kept at 25°C.

Discs *ex-vivo* culture

To image living wing discs we dissected the larvae in an ice-cold surface in a drop of PBS 1X. Discs were carefully picked without touching them with the dissecting tools to avoid tissue damage. They were placed in a petri dish containing 1mL of WM1 medium (Zartman et al.), ensuring that the tissues would be covered throughout the image acquisition and that nutrients would be sufficient. They were attached to the surface by placing them over a 1µL drop of poly-lysine solution. During the imaging period, temperature was controlled to mimic *in vivo* experimental conditions. Several temperature settings were tested to obtain an optimal disc performance along time and a proper control of gene expression trough temperature-sensitive constructs (see next section). In the experiments presented in this work the temperature of the microscope stage was 31°C, the air temperature was 33°C and the temperature of the objective was 35°C to avoid water condensation at the lens.

The *Gal4/UAS/Gal80* system

A very popular method used by *Drosophila* geneticists is the *Gal4/UAS* system (Brand and Perrimon, 1993). This permits the controlled expression of genes of interest to particular domains. The *Gal4* sequence, which encodes the yeast transcriptional activator Gal4, can either be cloned downstream a known promoter or inserted randomly in the genome downstream an endogenous enhancer. When Gal4 protein is produced it recognizes and binds to the *UAS* (Upstream Activation Sequence). We can clone the coding sequence of any gene of

interest using *UAS* as driver. Upon expression of *Gal4* in our particular domain, it will trigger the transcription of those genes downstream *UAS* sequences (figure MM1).

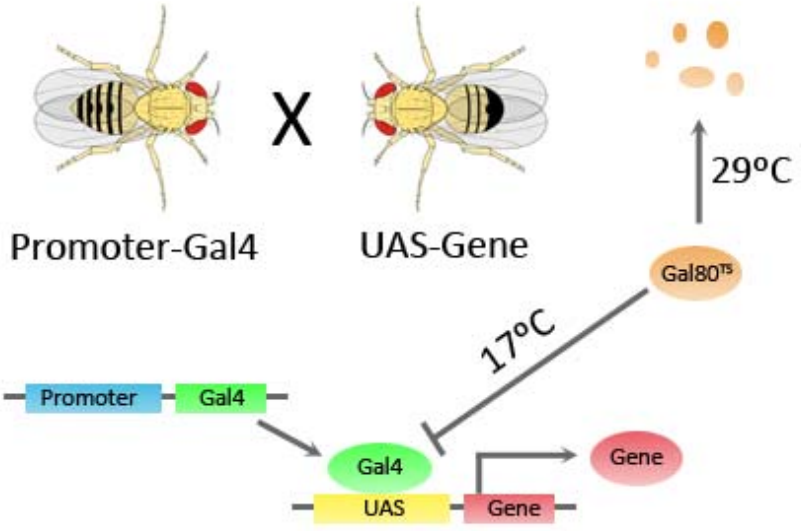


Figure MM1. The *Gal4/UAS/Gal80* system. Conditioned expression system widely used in *Drosophila*. The expression of a *Gal4* transcription factor is spatiotemporally controlled by a promoter of interest. Gal4 recognizes the *UAS* sequences and transcribes the gene associated to it. Having each transgene in different flies avoids unwanted expression of the UAS-Gene. If these flies are crossed, such expression is obtained. Gal4 activity can be modulated by its specific repressor Gal80. Gal80 has a temperature sensitive isoform ($Gal80^{TS}$) that is only active at low temperatures.

There is another level of spatio-temporal control of this targeted gene expression through the *Gal4*-inhibitor *Gal80*. If Gal80 is present, Gal4 is unable to bind to its target sequences. Else, there is an alternative form of Gal80 that is temperature sensitive (named $Gal80^{TS}$, (McGuire et al., 2003)). It is active at 17°C (and therefore Gal4 is not functional and there is no *UAS*-driven gene expression) and inactive at 29°C (Gal4 functions and induces the transcription of *UAS*-driven genes) (figure MM1).

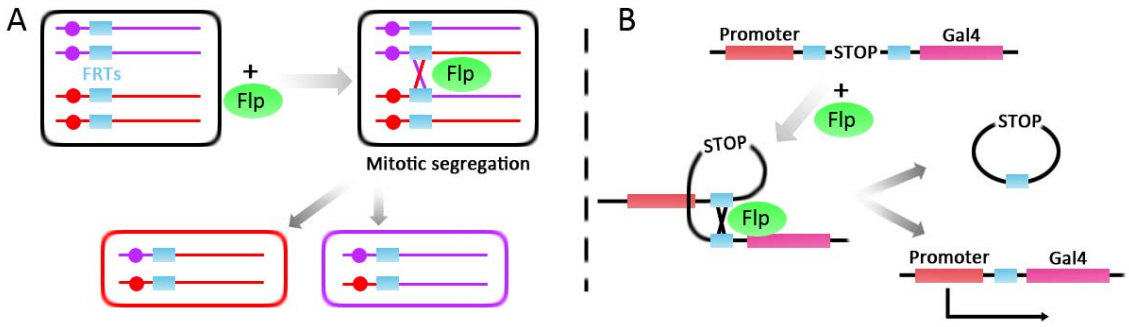
Clonal analysis

The FRT/Flp system

To assay the behaviour of a subset of cells that misexpress a gene (or genes) of interest growing within a normal tissue, we made use of the FRT/Flp system (Golic and Lindquist, 1989; Xu and Rubin, 1993). FRT (Flippase Recognition Target) sequences were isolated from yeast and are the target sequences for the yeast FLP recombinase.

If the FRT sequences are located at the same position of homologous chromosome arms, the Flp recombinase induces a *trans*-recombination at that position (figure MM2A). After a mitotic division, the resulting sister cells are genetically different.

If the FRT sequences are in the same chromosome arm, the Flp recombinase proceeds with the *cis*-recombination of these sequences (figure MM2B). This method is used to perform a controlled deletion of the sequence flanked by the FRTs.



FigureMM2. The FRT/Flp system. This system is based in the ability of Flp to recognize *FRT* sequences and induce the recombination between two of them. There are two possible settings: a trans-recombination in which the *FRTs* are located in the same position of homologous chromosome arms (**A**) and a cis- recombination in which the *FRTs* are located in the same chromosome arm (**B**). In the first case recombination induced in a heterozygous cell leads, upon mitotic segregation, to two sister cells genetically different. In the second setting recombination eliminates the DNA fragment flanked by the *FRTs*. In this example we have placed a STOP codon that blocks the transcription of a *Gal4* downstream a promoter. After recombination this expression can take place.

To control spatio-temporally the recombination of FRT sequences, we drive the expression of the Flp recombinase with different promoters. In this work we have used the Heat Shock Protein (HS) promoter (hs-Flp), which induces the expression of Flp upon an exposure to a high (over 34°C) temperature. To induce random clones in the tissue we heat shocked larvae for 7-15 minutes, as indicated in each experiment. To direct recombination to a concrete region, we used the *Gal4/UAS* system, using the *Gal4* driver to transcribe in the desired location a *UAS-Flp* (Struhl and Basler, 1993).

The MARCM system

To analyse the behaviour of clones that are mutant for a gene and that simultaneously express some cellular marker, other genes or dsRNAs against other genes, we made use of MARCM (Mosaic Analysis with a Repressible Cell Marker) system (Lee, 1999). This is a combination of the FRT/Flp and the Gal4/UAS/Gal80 systems.

The mutation of interest is placed in a chromosome arm distal to a *FRT* sequence. In the homologous chromosome arm Gal80 (Gal4 repressor) is located distal to a *FRT* sequence of the same location. A Flp-driven *trans* recombination and a mitotic event leads to two different sister cells: one bearing the mutation and Gal80-free (with a Gal4 source it will express any *UAS*-driven gene of interest) and the other one being wild type and having two copies of Gal80 (thus not expressing any *UAS*-driven sequence).

Fly stocks and genetic crosses

The *Drosophila* lines used in this study are:

- To study the behaviour of *rab5* mutant cells we used $rab5^{2}$, a null mutant that has a 4kbp deletion of the 5' part of the gene, containing the 5' UTR and the first exon of all the splice variants (Wucherpfennig et al., 2003). We crossed flies of genotype $rab5^{2}$ *FRT40A/CyO ; TM6B/MKRS* (or $rab5^{2}$ *FRT40A/CyO ; puc-lacZ/TM6B*) and *y w hs-Flp tub-Gal4 UAS-GFP ; tub-Gal80 FRT40A/CyO*. *puc-lacZ* from (Martin-Blanco et al., 1998).

- To generate a *rab5*-deficient posterior compartment we crossed *w ; If/CyO ; hh-Gal4 UAS-GFP/TM6B* (Tanimoto et al., 2000) and *y w ; If/CyO ; UAS-rab5 dsRNA/TM6B* ($rab5^{KD}$ - ID: 34096, Bloomington *Drosophila* Stock Center, BDSC) flies. Other dsRNAs against *rab5* were tested: BDSC, ID 30518 and Vienna Drosophila Resource Center, ID 102895.

- To produce $rab5^{KD}$ clones we crossed flies of genotype *y w ; arm-lacZ FRT40A/CyO ;* $rab5^{KD}$*/TM6B* (or *y w ; arm-lacZ FRT40A/CyO ;* $rab5^{KD}$ *puc-lacZ/TM6B*) and *y w hs-Flp tub-Gal4 UAS-GFP ; tub-Gal80 FRT40A/CyO*.

- To induce $rab5^{KD}$ Ras^{V12} clones we crossed *y w ; arm-lacZ FRT40A/CyO ;* $rab5^{KD}$ *UAS-*Ras^{V12}*/TM6B* flies to *y w hs-Flp tub-Gal4 UAS-GFP ; tub-Gal80 FRT40A/CyO* flies. *UAS-*Ras^{V12} from (Barbacid, 1987).

- To induce $rab5^{KD}$-expressing clones with a temporal control of its expression we crossed *y w hs-Flp ; + ; tub FRT GFP* y^{+} *FRT Gal4* and *y w ;* $tub\text{-}Gal80^{TS}$*/CyO ;* $rab5^{KD}$*/TM6B* flies.

- To drive the expression of $rab5^{KD}$ to different regions of the disc we crossed *y w ; If /CyO ;* $rab5^{KD}$*/TM6B* flies to flies carrying the specific drivers: *pannier-Gal4* (Calleja et al., 1996), *patched-Gal4* (Hinz et al., 1994), *nubbin-Gal4* (Calleja et al., 1996) or sal^{EPv}*-Gal4* (Cruz et al., 2009). sal^{EPv} is a fragment of the *sal* promoter that drives the expression to the central region of the wing.

- To induce *rab5^{KD}* expression to the *sal^{EPv}* domain with a temporal control of this expression, we crossed *y w ; If /CyO ; rab5^{KD}/TM6B* (or *y w ; If/CyO ; rab5^{KD} puc-lacZ/TM6B*) flies to *y w ; sal^{EPv}-Gal4/CyO ; tub-Gal80^{TS}/TM6B* flies.

- To trace the lineage of the *sal^{EPv}* domain we crossed *w ; sal^{EPv}-Gal4/SM5-TM6B/act- FRT FRT lacZ UAS-Flp* (or *w ; sal^{EPv}-Gal4/SM5-TM6B/ubi- FRT FRT GFP UAS-Flp*) and *y w ; tub-Gal80^{TS}/CyO ; rab5^{KD}/TM6B* flies. The GFP-marking cassette was a gift from C. Evans and U. Banerjee. The lac-Z-marking cassette is described in (Struhl and Basler, 1993).

- To perform *in vivo* analysis of growing tumours we crossed *y w ; sal^{EPv}-Gal4/CyO ; tub-Gal80^{TS}/TM6B* flies to *y w ; Histone2-RFP/SM5-TM6B/ rab5^{KD}* flies. H2-RFP flies were a gift from KD Irvine.

- To block apoptosis in the *sal^{EPv} rab5^{KD}* tumours we crossed *y w ; sal^{EPv}-Gal4/CyO ; tub-Gal80^{TS}/TM6B* flies and *y w ; dronc dsRNA/SM5-TM6B/ rab5^{KD}* flies. Also we crossed *y w ; sal^{EPv}-Gal4/CyO ; dronc^{I24} FRT80B/TM6B* flies to *y w ; If/CyO ; rab5^{KD} dronc^{I29}/TM6B*. *dronc dsRNA* obtained from the BDSC (ID 23035). *dronc* alleles are described in (Xu et al., 2005).

- To inhibit the JNK pathway in these tumours we crossed *y w ; sal^{EPv}-Gal4/CyO ; tub-Gal80^{TS}/TM6B* flies and *y w ; UAS-puckered^{14C}/SM5-TM6B/ rab5^{KD}* flies. The *UAS-puckered* line is described in (Martin-Blanco et al., 1998).

- To generate undead cells in the posterior compartment in a wild type *wingless* background, we crossed *w ; If/CyO ; hh-Gal4 UAS-GFP/TM6B* and *w ; If/CyO ; UAS-p35/TM6B* flies. The *UAS-p35* line was obtained from the BDSC.

- To generate undead cells in the posterior compartment in a *NRT-wg* background, we crossed *w ; NRT-wg/SM5-TM6B/hh-Gal4 UAS-GFP* and *w ; NRT-wg/SM5-TM6B/UAS-p35* flies. *NRT-wg* flies were a gift from JP Vincent, and described in (Alexandre et al., 2014).

- To perform the genetic screen we crossed flies of genotype *y w ; arm-lacZ FRT40A/CyO ; TM6B/MKRS* (for *arm-lacZ* control clones) or *lgl^{4} FRT40A/CyO ; TM6B/MKRS* (for *lgl^{4}* control clones) or *lgl^{4} FRT40A/CyO ; ds-RNA/TM6B* (for *lgl^{4}* clones that express a dsRNA against each candidate gene of Appendix table 1) with flies of genotype *y w hs-Flp tub-Gal4 UAS-GFP ; tub-Gal80 FRT40A/CyO*. The *lgl^{4}* allele was described as a null allele in (Mechler et al., 1985).

CyO, TM6B and SM5-TM6B are balancer chromosomes widely used by *Drosophila* geneticists to avoid homologous recombination. Those, together with If and MKRS are dominantly marked chromosomes used to follow chromosomes along genetic crosses.

X-rays treatment

To induce apoptosis, larvae were treated with a dose of 1500Rads. This corresponds to 100kV/15mA X-rays intensity during 3 minutes and 6 seconds at a distance of 18cm. This was performed in a Phillips-MG-102 device.

Immunohistochemistry

Antibody stain

To image fixed wing discs we dissected the larvae in an ice-cold surface and turned the body inside-out. The low temperature minimizes cellular stress and treatment-derived apoptotic cells. Larvae were fixed in a paraformaldehyde solution (4% in PBS 1X) with detergents (Triton X-100 0.1% and DOC 0.1%) for 1h at room temperature. After rinsing the fixing solution in washing buffer (PBS 1X, BSA 1% and Triton 0.1%) primary antibodies were incubated in washing buffer for 4h at room temperature or overnight at 4°C. After washing the primary antibodies secondary antibodies were incubated in washing buffer for 2h at room temperature and darkness. After washing the secondary antibodies discs were picked and mounted in Vectashield medium (Vector Laboratories). This medium reduces photobleaching due to laser incidence during image acquisition.

Following this protocol, different primary antibodies were used: rabbit anti-cleaved Caspase-3 (1:50; Cell Signalling Technology), rabbit anti-cleaved Dcp1 (1:200; Cell Signalling Technology), mouse anti-β-galactosidase (to detect -LacZ expressing cells; 1:50; Developmental Studies Hybridoma Bank), guinea pig anti-dMyc (1:100 generated in the laboratory by Francisco Martín, PhD), guinea anti-Yki (1:200; generated in our lab, see below), mouse anti-BrdU (1:10; Hybridoma Bank), rabbit anti-PH3 (M-phase of cell cycle; 1:100; Millipore), mouse anti-Mmp1 (1:50; Developmental Studies Hybridoma Bank), mouse anti-Wg (1:50; Developmental Studies Hybridoma Bank), rabbit anti-GFP (1:300; Invitrogen) and sheep anti-Digoxigenin-Alkaline Phosphatase (1:4000; Roche). Secondary antibodies were coupled to different fluorescent peptides (Alexa-488, Alexa-555 and Alexa-647). They were purchased from ThermoFisher Scientific.

To visualize DNA we used To-Pro-3 (ThermoFisher Scientific), named hereto after TOPRO. TOPRO was incubated, after rinsing the fixing solution, for 20 minutes in washing solution (1:500). Washing and mounting procedures were the same as described before.

To stain against filamentous actin we incubated discs with fluorophore-coupled Phalloidin, which binds specifically to F-actin: Phalloidin-TRITC (1:25; Sigma Aldrich) or Phalloidin-Alexa-647 (1:200; ThermoFisher Scientific).

BrdU incorporation

To detect cells in S-phase of the cell cycle we used a Bromodeoxyuridine (BrdU) labelling kit (Sigma-Aldrich). BrdU is an analogue of thymidine and can be incorporated by cells that are replicating their DNA.

Larvae were dissected in an ice-cold surface and incubated in a BrdU 0.01mM in PBS 1X for 1h at room temperature. After washing in PBS 1X they were fixed in paraformaldehyde 4%. After rinsing with PBS 1X, and to permit the interaction between the BrdU chemical and the antibody, they were treated with RQ DNAse (Promega) for 2h at 37°C. After washing the larvae, they were incubated with antibodies as previously described.

TR-Dextran incorporation

To assess the endocytic activity of the cells we dissected the larvae in an ice-cold surface and incubated in a Texas Red-Dextran (TR-Dextran; lysine fixable, MW3000, Molecular Probes) solution (2.2% in Shield and Sang's M3 medium, used to culture living tissues) in darkness. TR-Dextran is a sugar molecule conjugated with a red fluorophore that gets easily stuck to the cell surface. Upon interaction cells tend to intake and process it by the endocytic machinery. A pulse-chase assay is performed to observe if TR-Dextran has been endocytosed or remains in the surface of the cells. In our experiments the pulse period was 10 minutes at room temperature and, after rinsing the larvae in M3 medium, a 1h chase period was given. After this, the normal procedure was followed to fix and stain with antibodies when needed.

in situ *hybridization – antibody double stain*

We optimized a procedure from three available protocols to stain against antibodies and hybridize the same sample with a RNA probe. To achieve this we dissected larvae in an ice-cold surface and fixed them in 4% paraformaldehyde for 30 minutes at room temperature. A second fixation was performed adding Tween-20 0.1% to the paraformaldehyde 4% for 30 minutes. We rinsed for 5 minutes in sequential dilutions of PBT-Tween-20 0.1%/Hybridization Solution (**HS**): 70/30-50/50-30/70-HS 100%. Larvae were kept in HS for 30 minutes to several days at -20°C. Pre-hybridization consisted on permeabilizing cell membranes by heating the larvae for 2h at 55°C. After that the denatured Digoxigenin-labelled probe is incubated with the tissue overnight in HS at 55°C. Rinsing is performed with warm HS and larvae are

rehydrated in sequential solutions of HS/PBT-Tween-20 0.1%. Incubation with the primary antibody anti-Digoxigenin conjugated to Alkaline Phosphatase was performed overnight at 4°C. Other primary antibodies were added too. Fluorescent proteins are bleached during the procedure, so antibodies against them were used when necessary. After washing the antibodies with PBT-Tween-20 0.1%, secondary antibodies were used if needed. After that we incubated the larvae in a solution (**SB**) with substrates for alkaline phosphatase (NBT and BCIP) that provoke a chromogenic reaction, developing a black-purple signal that reveals the localization of the RNA probe in the tissue.

HS: 50% formamide, SSC 5X, 64µg/mL tRNA, 50µg/mL heparin, Tween-20 0.1%.

SB: NaCl 4M 2.5%, MgCl 1M 5%, Tris 1M pH 9.5 10% and Tween-20 0.1% in dH_2O.

Antibody generation

We generated a polyclonal antibody against the protein Yki. For that purpose we obtained a vector that contained a full length Yki fused to GST (at the SmaI site in a pGEX-3X vector, Amersham Biosciences)(Oh and Irvine, 2008). We transformed the plasmid into BL21 (DE3) *E. coli* (Invitrogen) by electroporation using the manufacturer protocol. We grew the bacteria in LB medium with ampicillin (100µg/mL) up to an optical density of 0.6 (spectrophotometer λ=595nm) which corresponds to approximately 0.6 g/L of dry cells. Yki expression was then induced by IPTG 1mM during 2.5h. Purification of the protein was done by isolating the appropriate gel bands, which were mixed and emulsified (1:1) with Freund's complete adjuvant (Sigma Aldrich) for the first dose and with Freund's incomplete adjuvant (Sigma Aldrich) for the reminder doses. One guinea pig was immunized by injecting it about 300µg of the protein the first time and 150µg in 4 reminder injections.

Acquisition and image analysis

Fixed tissue imaging was performed in a Leica TCS SPE confocal microscope (Leica Microsystems). Tissues treated for in situ hybridization - double stain were imaged using a Zeiss LSM510 confocal microscope (Zeiss). Live imaging was performed with a Perkin Elmer Ultraview spinning disc confocal microscope (Perkin Elmer). During live imaging chamber temperature was controlled.

Image processing and analysis were performed with Photoshop CS4 (Adobe Systems) and ImageJ/Fiji (NIH) programs. To obtain the P/A ratio and coverage of a region of interest (domains, clones, compartments…) in a disc, macro sequences were designed in Fiji to

measure all the samples avoiding human errors. To plot dMyc protein levels of a region, a selection was made (square in figure R9) and a histogram was generated by merging the grey values of the data. To generate the orthogonal sections of a stack of images (figure R12) Fiji was used.

Statistical analysis

Throughout the thesis work we present several quantitative analyses of confocal images. To obtain the numerical data we processed images with macro programs in Fiji. These data were further analysed and treated using Microsoft Office Excel 2007.

Unless otherwise indicated, standard deviation of the *n* samples was calculated to obtain the standard error of the mean of each data batch. To compare experimental conditions to their controls, we used the two-tailed Student's *t*-test type 3. In the histograms means are plotted and standard error bars are represented. Statistical significance of the comparisons is represented by asterisks, meaning: *=*P*-value < 0.05 and **=*P*-value < 0.01.

RESULTS

Usage of *rab5* mutants as a model to study cell competition

rab5 mutant cells are eliminated in a wild type tissue

It has been proved that null mutations in *rab5* induce neoplastic transformations of tissues: they overgrow, show disorganized architecture, cells lose their apico-basal polarity and fail to undergo final differentiation (Lu and Bilder, 2005).

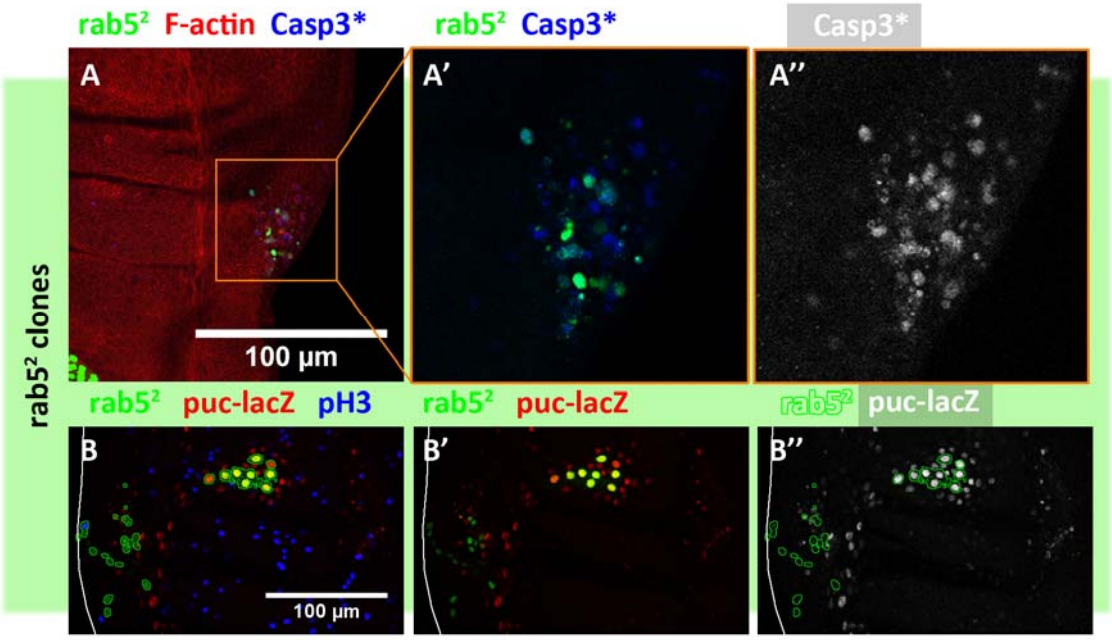


Figure R1. *rab5* mutant cells activate JNK and die through apoptosis in a mosaic tissue. GFP-marked clones of mutant cells for *rab5* were induced in a wild type tissue and observed after 96h (panel **A**) or 48h (panel **B**). High magnification of representative clones is shown. These cells are specifically marked by the presence of activated-Caspase3 (blue, **A**), which indicates activity of the apoptotic machinery. Additionally, *puckered-lacZ* (*puc-lacZ*) reporter activation indicates that the Jun-N terminal Kinase pathway could also play a role in the elimination of these mutant clones (**B**). In **B''** we delineate the outline of the GFP-marked cells to better observe the co-localization of the *lac-Z* signal.

Here, we have studied the behaviour of *rab5*^{2} mutant cells in the *Drosophila* wing imaginal disc. These mosaic tissues have been obtained by inducing homozygous *rab5*^{2} clones in a *rab5*^{2}/+ background using the FRT/Flp system (Golic and Lindquist, 1989; Xu and Rubin, 1993). Clones are marked by gain of GFP with the MARCM system (Lee, 1999) (see Materials and Methods). In figure R1 isolated *rab5*^{2} mutant clones generated in the wing disc are shown at high magnification. These clones tend to be small as compared with control non-mutant clones, indicating a low proliferation rate, cell death or both. *rab5*^{2} clones grow normally for up to 48 hours but then enter apoptosis, as visualized by staining of active Caspase3 (figure R1A). As a result, 96 hours after clone induction, very few discs show *rab5*^{2} clones (less than control discs), indicating that they died through apoptosis. Interestingly, we also observed that the majority of these clones activate the Jun N-terminal Kinase (JNK) pathway, as seen by the reporter *puckered-lacZ* (*puc-lacZ*), a target and a negative regulator of the pathway (figure R1B). Altogether, these results suggest that *rab5* mutant cells are lost due to cell competition (Morata and Ripoll, 1975), as they are viable when in a homotypic environment, but die when confronted with wild type cells.

rab5^{KD} can induce neoplastic overgrowths

In addition to the *rab5*^{2} mutation, we used two different dsRNAs against *rab5* (see Materials and Methods). Both resulted in a blockage of endocytosis in the affected cells. The endocytic function of the cells can be assayed by the incubation of the living tissue with Texas Red-Dextran, which is accumulated on the cell surface if endocytosis is impaired. A detailed protocol for this assay is found in Materials and Methods.

The phenotypes of the dsRNAs and the *rab5*^{2} mutation are comparable in many aspects and since the usage of UAS-driven transgenes offer operational advantages, we decided to continue our work using a dsRNA against *rab5*. More specifically, in this work we present the results obtained with the UAS-*rab5 ds-RNA* (ID: 34096), to which we refer to as *rab5*^{KD}.

First we expressed *rab5*^{KD} in the posterior compartments of the larvae, which are lineage-restricted independent growth units, by using a Gal4 line driven by the *hedgehog* promoter (*hh-Gal4*). Moreover, we controlled the temporal expression of *rab5*^{KD} by using the temperature-sensitive Gal4 repressor Gal80^{TS} (McGuire et al., 2003), which is active at the temperature of 17°C but degraded at 29°C (see Materials and Methods). We could already observe phenotypes associated to the lack of endocytosis 16 hours after shifting the larvae to 29°C to inactivate Gal80^{TS} and therefore promote *Gal4* expression and abolish *rab5* function. In figure R2 we show the evolution of the wing discs after the temperature shift. We observe that

the *rab5*^{KD} posterior compartments gradually increase, reaching a size significantly bigger than the control discs. We quantified this overgrowth by measuring the posterior-to-anterior surface ratio (P/A ratio), and the increase in this parameter is clearly visualized in the graph (figure R2E). Associated to the *rab5*^{KD} phenotype we found high levels of apoptosis within the posterior compartment, detected with an antibody against the caspase Dcp1.

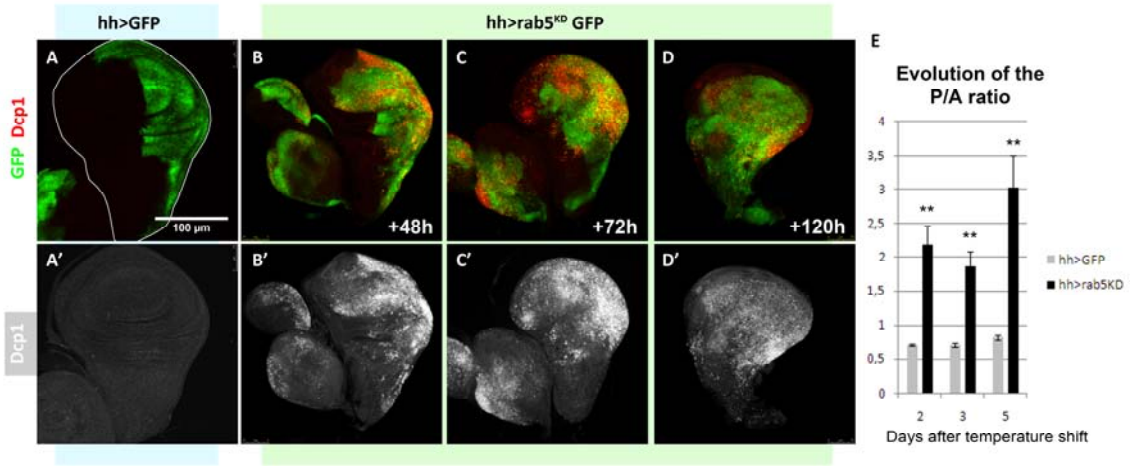


Figure R2. *rab5*^{KD} induces neoplastic overgrowths when expressed in the posterior compartment. According to the neoplastic phenotypes associated to the lack of *rab5* function using mutant alleles (Lu and Bilder, 2005), the expression of a dsRNA against *rab5* (*rab5*^{KD}) in an entire compartment triggers the unrestricted growth of the tissue. The evolution of these compartments is shown in panels **B-D** and compared to control GFP-expressing disc in panel **A**. Associated to *rab5*^{KD} expression we can observe autonomous cell death by the presence of the activated effector caspase Dcp1 (**A'-D'**). We measured the relative size of the posterior (P) and anterior (A) compartments both in control and experimental discs (panel **E**).

We conclude that a compartment entirely comprised of *rab5*^{KD} cells acquires an oncogenic character, as indicated by its unrestricted growth and abnormal cell differentiation.

rab5^{KD} cells are eliminated in a mosaic tissue

To validate the usage of *rab5*^{KD} cells as a cell competition model we created mosaic tissues in which clones of *rab5*^{KD} cells are confronted to wild type cells. Subsequently, we analysed the behaviour of the *rab5*-deficient clones. Like in the analysis of *rab5*^{2}, *rab5*^{KD} clones survive for 48 hours after initiation, with size and shape comparable to control GFP-expressing clones. At that time there is no Caspase 3 or Dcp1 staining within the clones. However, 72 hrs after clone initiation onwards we can observe Caspase 3 staining in the clones, which means that these cells are being eliminated from the tissue (figure R3B). As a landmark of cell competition, we checked whether the JNK pathway was also active in these cells. Indeed this was the case, as visualized by the *puc-lacZ* reporter (figure R3C).

Altogether, we conclude that *rab5*^{KD} cells are a convenient material to study the role of cell competition in tumorigenesis.

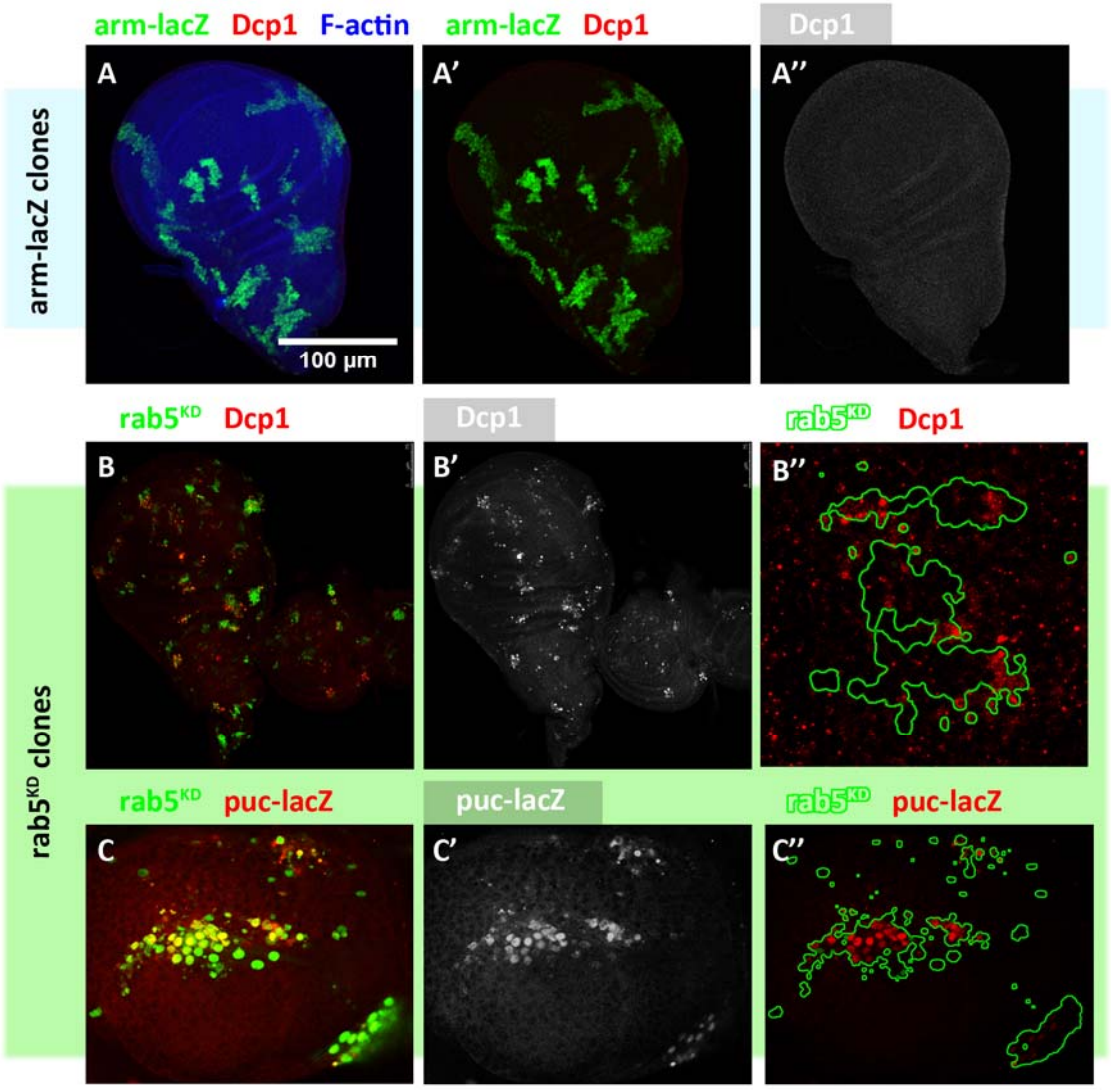


Figure R3. Clones deficient for *rab5* are specifically eliminated when growing within a wild type tissue. The behavior of *rab5*^{KD} clones resembles that of the *rab5*^{2} mutant clones when induced in a mosaic tissue. In panel **A** we show a wild type disc with wild type GFP-expressing clones induced 72h before fixation. No Dcp1 activity is observed in these conditions (**A''**). However, after 72h of *rab5*^{KD} expression we recover discs in which clones are disaggregated and clearly smaller than control clones (**B**). Dcp1 activity is observed in most of these clones indicating apoptotic cells. *puc-lacZ* signal is also observed, indicating JNK activity. Clone outline is shown in **B''** and **C''** for the ease of observation. Scale bar in **A** stands for **A-A''** and **B-B'**. **B''** and **C** are high magnifications of individual clones. Clones of all images were induced by a 15' heat shock treatment.

Microenvironment and cell competition

It was previously proposed that clones mutant for the tumour suppressor gene *lgl*, normally doomed to die by cell competition, may be able to survive and cause overgrowths when merged with other mutant clones (Menendez et al., 2010). Clone merging creates a protective microenvironment that allows inner cells to escape the anti-tumour role of cell competition. However, definitive proof of this proposal was lacking. Also, there was no estimate of the size of the microenvironment required to escape cell competition. Here we present a series of experiments addressing these questions.

Groups of cells deficient for *rab5* may survive if a critical mass is reached

We show in figures R1 and R3 that clones deficient for *rab5* in a wild type context are rapidly eliminated by cell competition. However, to study the neoplastic potential of *rab5*-deficient cells we provided them with a proliferative advantage, a method previously used with other tumour suppressor genes in *Drosophila* (Brumby and Richardson, 2003; Menendez et al., 2010; Pagliarini and Xu, 2003). To this purpose, we co-expressed $rab5^{KD}$ and a constitutively active allele of *Ras1*, Ras^{V12}, using the UAS-Ras^{V12} transgene. The use of this allele ensures the permanent activity of the Ras pathway, which induces the expression of a broad range of target genes responsible for a high metabolic status in the cells.

We studied the behaviour of these clones in two different contexts: 1) few clones per disc - an average of 2-3 clones per disc obtained by treating the organism with a short heat shock (7 minutes), and 2) numerous clones per disc - about 23-24 clones per disc, induced by heat shocking for a longer time (15 minutes).

In the first experimental setting $rab5^{KD}$ Ras^{V12} clones may be found up to four days after clone induction. This is an increase in survival of about one day compared to $rab5^{KD}$ clones. However, even though these clones are protected from apoptosis by the downregulation of the proapoptotic gene *hid* downstream of Ras (Bergmann et al., 1998; Kurada and White, 1998), we can detect apoptotic cells in the clones (figure R4B-C). This is important as it suggests that cell competition is occurring independently of a metabolic status-comparison between the two confronting cell types. It suggests that cells might be comparing cell identities, and the difference would be the trigger for cell competition.

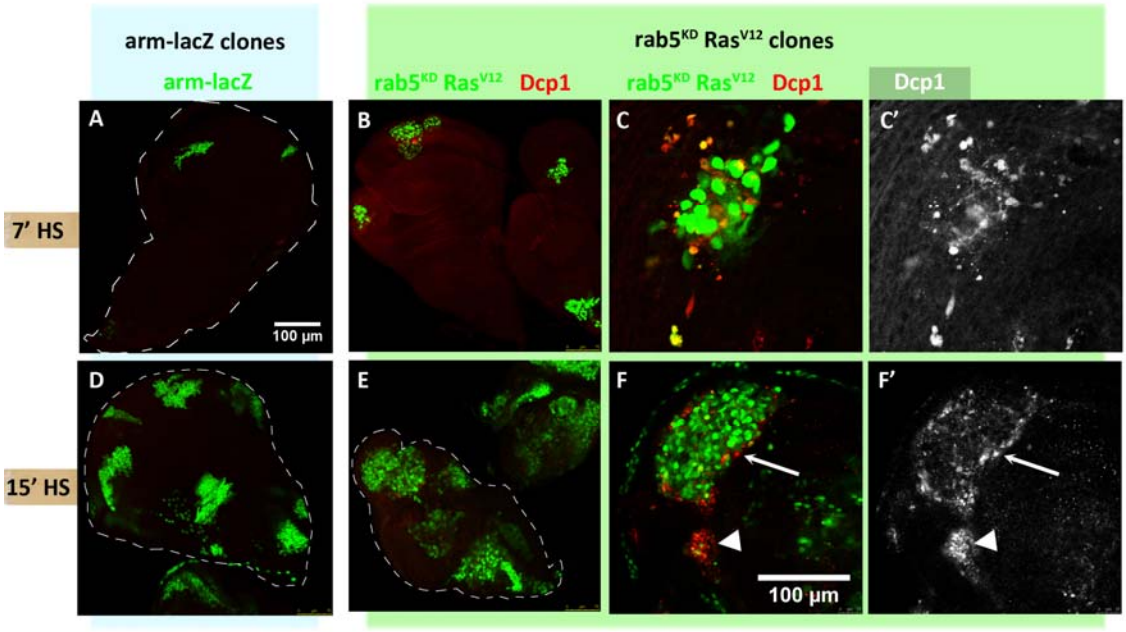


Figure R4. Different behavior of *rab5^{KD} Ras^{V12}* clones depending on the clone density in the disc. To induce different clone densities we treated the larvae with 7 (**A-C**) or 15 (**D-F**) minutes of heat shock (HS) time. In **B-C** we can observe that these clones activate Dcp1 and tend to disappear from the tissue as compared to control clones in **A**. Discs fixed 72h after clone induction. At later stages very small clones are recovered. Instead, a long HS treatment increases the proportion of discs with overgrowing patches of *rab5^{KD} Ras^{V12}* cells (**E**). Still, Dcp1 is activated at the periphery of the large clones (**F** arrow) or in entire small patches that are eventually eliminated (**F** arrowhead). Discs fixed 144h after clone induction. Scale bar in **A** stands for **A**, **B**, **D** and **E**. **C** is a high magnification of an individual clone.

In the experimental setting in which a high number of clones are induced in the disc, the behaviour of the clones is different. The Dcp1 staining indicates that *rab5^{KD} Ras^{V12}* cells still die when in contact with the wild type surrounding cells. However, the area occupied by *rab5^{KD}* clones is clearly higher than in the previous cases, and we can find discs with big patches long time after clone induction (figure R4E and R5). When analysed in detail, however, we find small clones that are often marked completely by Dcp1 staining (figure R4F, arrowhead), meaning that they are actively eliminated from the tissue. Apoptotic cells can also be observed at the periphery of the big patches of cells (figure R4F, arrow), suggesting that they are identified and targeted for death by cell competition. However, in spite of their peripheral cells undergoing apoptosis, these big patches tend to overgrow and occupy the entire disc (figure R5). We quantified the number of independent patches of *rab5^{KD} Ras^{V12}* cells, and found an inverse correlation between this number and tumorous status of the disc (figure R5G), pointing to the idea that a proportion of the clones merge to form a tumour, while others (likely small,

isolated ones) are eliminated by cell competition. The overgrowing *rab5^{KD} Ras^{V12}* patches acquire a multi-layered and disorganized structure, indicating a neoplastic transformation.

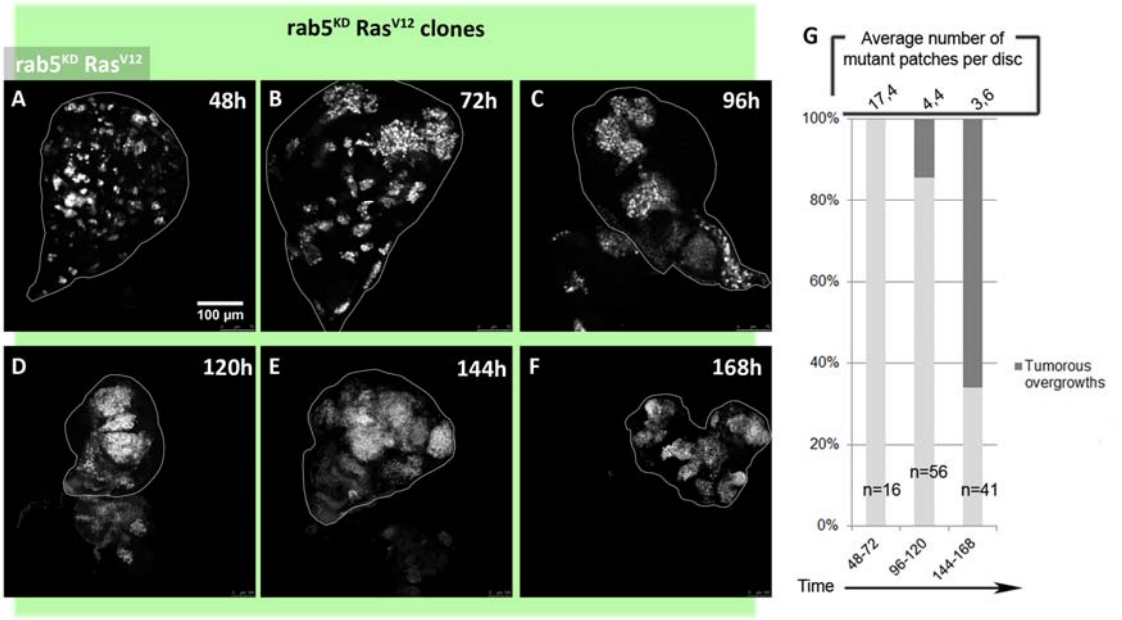


Figure R5. *rab5^{KD} Ras^{V12}* clones induced in a high density context tend to merge and overgrow. In panels **A-F** we can observe the evolution of the coverage of the *rab5^{KD} Ras^{V12}* patches in the disc from 2 to 7 days after clone induction. Disc outline is depicted in all panels. We quantified this coverage and considered it to be a tumorous overgrowth when it reached over 50% of the disc (**G**).

Altogether, these data and previous work (Menendez et al., 2010), strongly suggest that oncogenic cells subject to cell competition can evade this tumour suppressing mechanism by merging into large patches. Because cell competition is a short-range process that takes place between cells in close contact, it eliminates cells at the borders of the tumours, but cells inside the big patches are beyond the reach of cell competition. This phenomenon creates a protective microenvironment that gives central cells the opportunity to proliferate and fomr a tumour. If a patch of cells does not reach a critical mass, it will eventually disappear, as the rate of cell elimination at the periphery would overrule the rate of proliferation at the center. The two opposite destinies that a patch can follow depending on its size is further discussed and represented in figure D1 (Discussion section).

A second approach to study the role of a microenvironment to evade cell competition is to induce wild type clones in a wild type context, which will grow normally, and transform them into *rab5^{KD}*-expressing clones after they have reached a certain size. This can be achieved with a *tubulin FRT GFP (stop) FRT Gal4* cassette. With a short Flp pulse the sequence flanked by

FRTs is flipped-out and constitutive expression of GFP is randomly replaced by expression of Gal4 (see schematic example in figure R6E and MM2B). Temporal expression of *rab5*^{KD} is controlled by the Gal80^{TS} tool that represses Gal4 activity at 17°C, but not at 29°C (see Materials and Methods and figure MM1). Thus, a temperature shift from 17°C to 29°C transforms the clone cells from normal to oncogenic (figure R6). By using this system we allow the clones to grow to a large size before they are transformed into *rab5*^{KD} and cell competition starts. As a control we kept larvae at 17°C, avoiding the expression of *rab5*^{KD}, and incubated the discs with Dextran-Texas Red (figure R6A). Cells did not accumulate Dextran at the surface, indicating that they were not deficient for *rab5* function. Also, a control without the *rab5*^{KD} transgene was used and comparable results were obtained. *rab5*^{KD} clones accumulated Dextran at the cell surface, as shown in figure R6C.

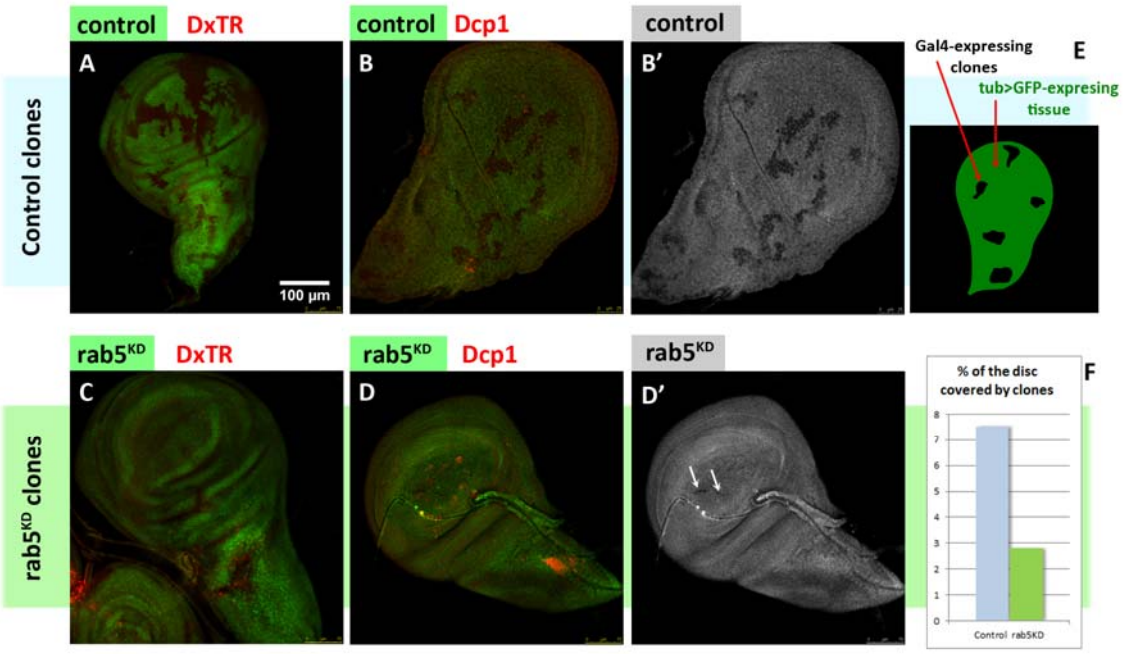


Figure R6. Relatively large clones transformed by *rab5*^{KD} expression fail to overgrow. Flp-out Gal4-expressing clones were induced and, using Gal80^{TS}, a temperature shift transformed them into *rab5*^{KD}-expressing clones in experimental discs (**C-D**). Control clones were not subject to the temperature shift (**A-B**). Clones are marked by the lack of GFP (schematic example in **E**). Control clones do not show accumulation of Dextran-Texas Red (DextranTR) (**A**), while experimental disc did (**C**). Clone size decreased and Dcp1 activity was found when *rab5*^{KD} expression was induced. Quantification of the clones' coverage over the discs was done and represented in **F**.

We found that in very rare cases did *rab5*^{KD} patches overgrow; instead, they were generally eliminated. It is worth noting that few clones were induced to avoid clone merging. In the

graph in figure R6F we show that the proportion of the discs covered by the GFP^- clones is significantly smaller when they express $rab5^{KD}$.

However, we consider this was not a good system to study the mechanisms that allow tumours to overcome cell competition. Even though we obtained big clones expressing $rab5^{KD}$, due to their irregular shape by the time the $rab5^{KD}$ expression was induced, a large proportion of their cells were exposed to wild type cells, preventing the generation of a microenvironment.

Finally, we decided to drive the expression of $rab5^{KD}$ in defined domains of different dimensions and shapes within de disc. In these experiments we could follow the potential oncogenic transformation of groups of cells.

1. We used the *pannier*-Gal4 driver, which is expressed along the dorsal central domain of the organism. In the wing disc it is restricted to the proximal region, which will form the central notum (see figure R7A). The expression in this domain of $rab5^{KD}$ does not create a tumorous overgrowth. Instead, the discs have a reduced dorsal region (figure R7A'). This suggests that the notum – which is part of the body trunk – is less prone or refractory to undergo oncogenic transformations.
2. *patched*-Gal4 drives *Gal4* expression in an anterior band of cells along the antero-posterior boundary of the wing imaginal disc (figure R7B). The band is narrow, so when we expressed $rab5^{KD}$ using this driver a big proportion of the transformed cells are exposed to cell competition while only a few were protected. Again, no overgrowths were found in the presumptive notum region, while in the hinge or in the wing blade (the prospective appendage domain) we could find slight overgrowths in a few cases (figure R7B').
3. Next we studied the behaviour of transformed domains of the appendage region. By using *nubbin*-Gal4, we drove the expression of $rab5^{KD}$ to the entire prospective wing and haltere (thus in the haltere disc, homologous to the wing one). As shown in figure R7C', the size and location of this domain is sufficient to generate severe overgrowths and epithelial structure loss. Tissue folds and cell multi-layering can also be observed using confocal microscopy.

   Similar results were obtained using the sal^{EPv}-Gal4 driver. Its expression is restricted to the wing blade primordium (figure R7D). Although the size of this domain is smaller than the *nubbin* region, it appears to be sufficient for the transformed cells to overcome cell loss at the border due to cell competition, and overgrow (figure R7D').

The study of these different expression domains gave us a general view of the behaviour of tumour cells in different parts of the disc. As a first conclusion, we have found that, due to still unknown mechanisms, the notum region does not facilitate the generation of overgrowths. Interestingly, the appendage domains (targeted by the *nubbin* and sal^{EPv} *Gal4* lines) are more prone to overgrow and display the neoplastic traits that characterize the absence of *rab5*. As a second conclusion, we can determine that the smallest domain that gave rise to an overgrowing tumour was sal^{EPv}. Therefore, we selected this driver for the forthcoming experiments. Through further analysis using $Gal80^{TS}$ we estimated that the size of the domain by the time we induce the transformation would be of about 400 cells, arranged in a square-like shape, which facilitates the generation of a protective microenvironment.

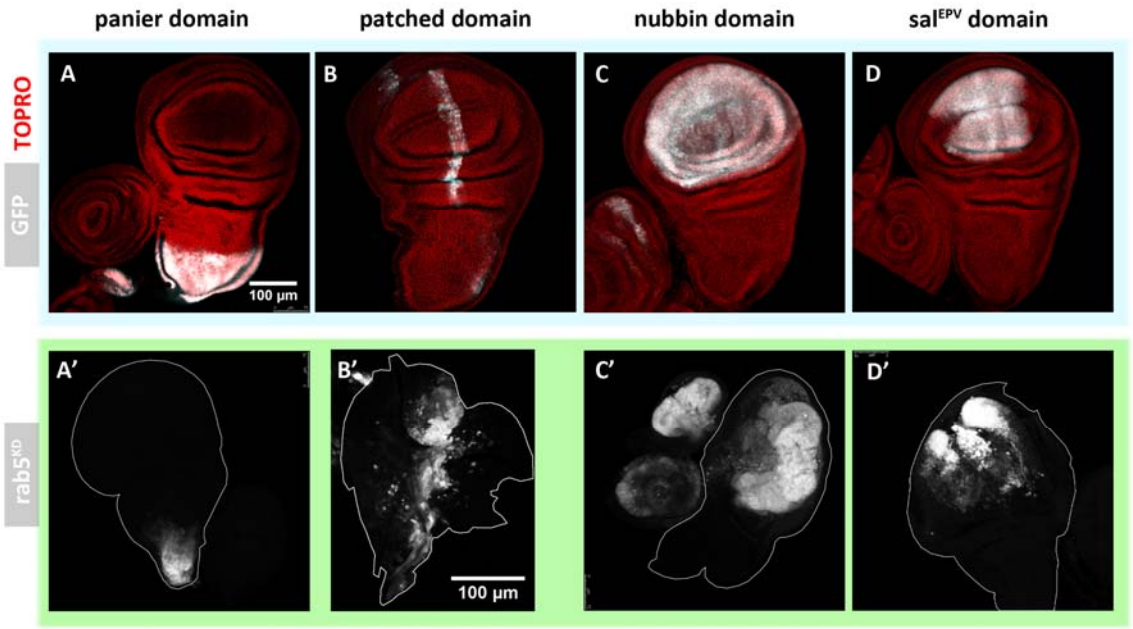


Figure R7. Expression of $rab5^{KD}$ in different domains of the disc. We observe different behavior of rab5 deficient cells depending on the transformed domain. Control discs (**A-D**) express GFP, while experimental (**A'-D'**) co-express GFP and $rab5^{KD}$. Scale bar in **A** stands for all images but **B'**, which has its own scale bar.

Role of cell competition-associated apoptosis on tumour growth

We have shown that the confrontation of $rab5^{KD}$ and wild type cells triggers apoptosis of the $rab5^{KD}$ cells even though these are viable and can generate overgrowths when in a homotypic environment. This apoptosis therefore functions as a tumour suppressing mechanism, removing oncogenic cells from the epithelium. In this section we present several experiments that suggest a dual role for apoptosis in tumour development.

Evolution of the pattern of apoptosis

We induced the expression of *rab5*^{KD} in the *sal*^{EPv} domain, marked by GFP, and temporally modulated by Gal80^{TS}. When *rab5* function was interfered for one day there was no evident tumorous transformation of the tissue although an increase in size was detected (figure R8B). The shape and tissue organization of the domain was comparable to the wild type control in which only GFP was expressed (figure R8A). However, we could observe high levels of Dcp1 homogeneously distributed throughout the domain (figure R8B'). This phenotype could already be observed 16 hours after the temperature shift (not shown).

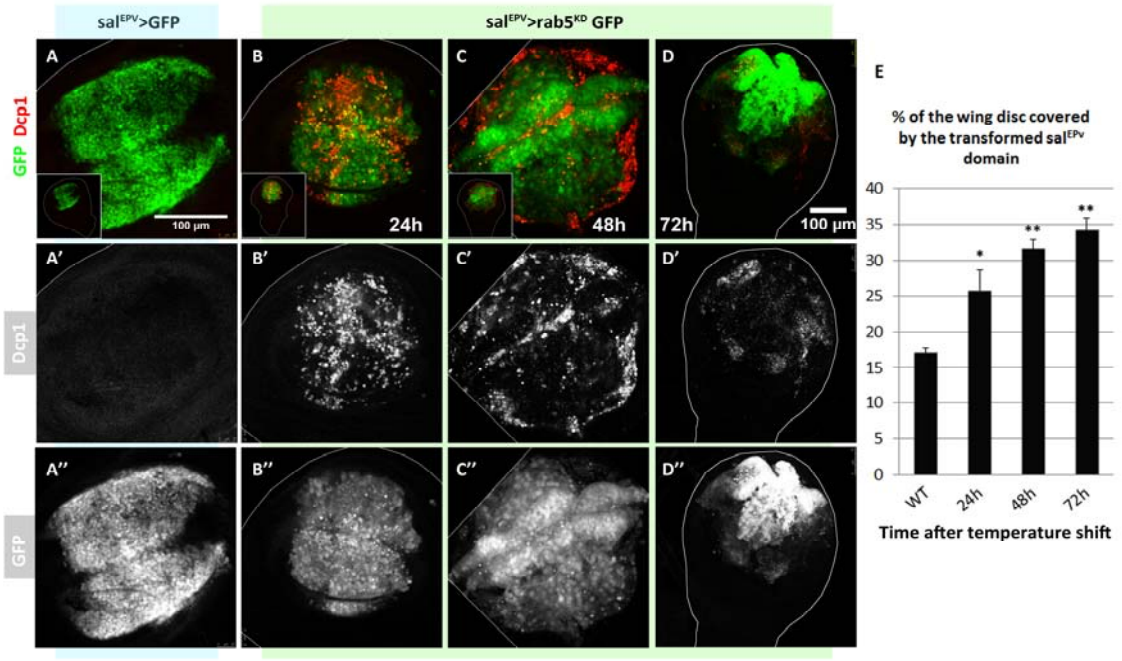


Figure R8. Evolution of the transformed *sal*^{EPv} domain over time. rab5KD was expressed in the *sal*^{EPv} domain (**B-D**) and compared to a GFP-expressing domain (**A**). Apoptosis has a stereotyped pattern that changes over time (**B´-D´**) when rab5 function is abolished. The relative size of the *sal*^{EPv} domain over the disc was quantified and is presented in **E**. Statistical comparisons have been done between GFP-expressing (WT) discs and each time point. Discs' border is outlined in all images. Scale bar in **A** stands for **A-C** and are high magnifications of their corresponding insets. *sal*^{EPv} domain in **D** is too large to fit in a picture of the same scale as **A-C**.

If the *rab5*^{KD} condition is sustained over time the domain suffers strong changes in size and shape. After 48 hours of *rab5* inactivation there still are apoptotic cells homogeneously distributed, but a new feature of the apoptosis pattern appears at this time. A clear band of GFP-positive cells shows Dcp1 staining surrounding the domain (figure R8C). There is supporting evidence that this apoptosis is due to cell competition: it does not appear until the

two cell types have been confronted for at least 48 hours and it is restricted to tumour cells at the border of the tumour.

Interestingly, one more day of expression of the transgene induces a neoplastic-like transformation of the domain, which acquires aberrant and irregular shapes as well as cell multi-layering. By this time the homogeneous apoptosis inside the domain is less patent, but the border cells show high levels of Dcp1 (figure R8D).

We quantified the proportion of the wing disc that was occupied by the transformed domain and compared it to the GFP control. The results are shown in figure R8E, where we can observe a gradual increase in the proportion of the discs covered by the tumour tissue.

The expression of *rab5*^{KD} in the *sal*^{EPv} domain triggers a delay in pupariation of about 2-4 days compared to control larvae. This is a recurrent phenotype of larvae carrying neoplastic tumours.

Response of the wild type surrounding tissue to the tumour

Most of the studies on tumour growth have focused their attention to the phenomena that occur in the tumour cells, ignoring the possible repercussion that their presence might have on the surrounding tissue. However, organs and tissues are known to react to tissue damage and other insults that may occur during development. Therefore, we studied how the cells surrounding the tumour react to it.

The previously reported activity of Dcp1 and other protein patterns like that of F-actin, DE-Cadherin or TOPRO lead us to conclude that these cells are healthy and that no signs of cell death, loss of polarity or tissue architecture are detected (not shown). Interestingly, however, the metabolic status of wild type cells change upon tumour induction in their vicinity.

A good reporter of the metabolic situation of a cell is dMyc, a downstream target of several signalling pathways that positively regulate cell proliferation and survival. dMyc is a transcription factor and regulates ribosome biogenesis, metabolism and translation (reviewed in (Conacci-Sorrell et al., 2014)). The staining of the dMyc protein in a wild type disc reveals a two-fold increase of its levels in the wing blade primordium that does not correspond to an increase of proliferation (figure R9A'', plot). When *rab5*^{KD} is expressed in the *sal*^{EPv} domain for three or more days, we observe a 4-fold increase in dMyc levels in the GFP^{-} wild type cells surrounding the tumour cells, while the latter show a reduced staining (figure R9B, plot). Interestingly, co-incubation with anti-Dcp1 shows that cells that are surrounding groups of dying cells are the ones that have the most increased levels of dMyc (figure R9B'').

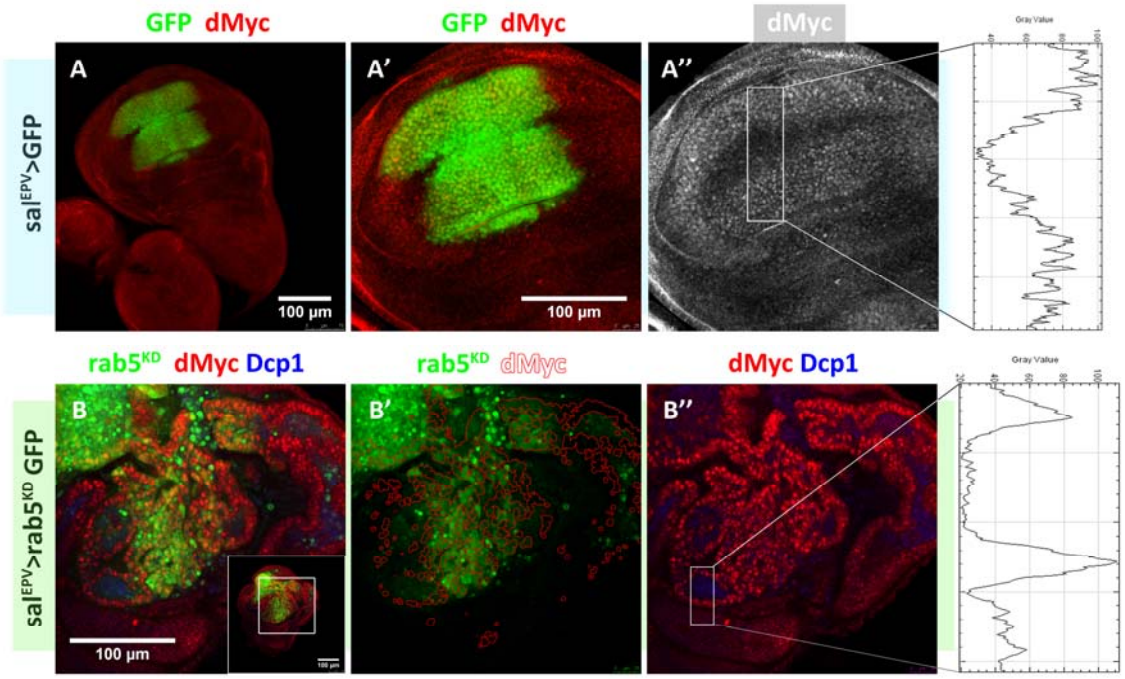


Figure R9. dMyc expression levels change upon tumour induction. dMyc has a heterogeneous pattern in a wild type disc in which GFP is expressed in the *sal*^{EPv} domain (**A**). If we induce the *rab5*^{KD} transformation of the domain, dMyc pattern suffers strong changes. A magnification of a tumour border is shown in **B** (entire disc shown in the inset). We have outlined cells with high nuclear dMyc signal in **B'** to show that GFP-expressing cells usually have low levels of dMyc. We have plotted the grey value of dMyc signal of the depicted sections of **A''** and **B''**. Grey values of these images range from 0 to 255, being 0 lack of dMyc protein, and 250 saturation of the signal. Note that plots are vertically comparable as axis values have been lined up.

As a double check, we also examined the activity of the Hippo pathway. This consists of a kinase cascade that leads to the phosphorylation of Yki, a transcription factor that is therefore retained in the cytoplasm where it does not have any known function. If the pathway is inactive, Yki is translocated to the nucleus, where it promotes the transcription of downstream targets that will, in turn, enhance survival and proliferation of the cells (reviewed in (Halder and Johnson, 2010)). We checked the protein levels and the subcellular localisation of the protein Yki in experimental and control discs (figure R10). Control discs show a ubiquitous presence of Yki, with a clear cytoplasmic localisation (figure R10A''), indicating an active status of the Hippo pathway. However, in the surroundings of a *rab5*^{KD} tumour induced in the *sal*^{EPv} domain, we observed a local increase of the protein signal (figure R10B'') and a more diffuse subcellular localisation of it, meaning it could be both cytoplasmic and nuclear. This indicates that wild type cells could be activating dMyc at the periphery of the tumours by increasing the amount and the activity of Yki.

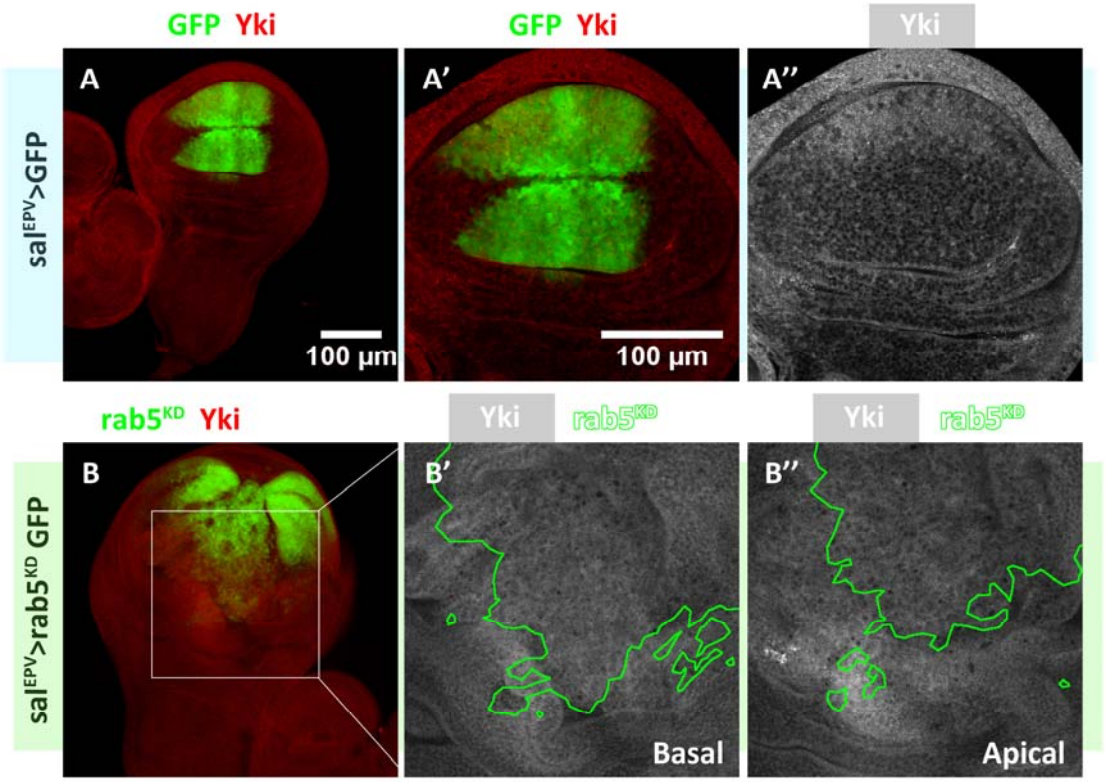


Figure R10. Yki levels increase next to the tumour border. Wild type discs show relatively low levels of Yki, although homogeneously distributed, as visualized through antibody staining (**A**). However, in experimental discs with tumorous transformation of the *sal*^{EPv} domain we observe a slight increase of Yki in areas in which apoptotic cells are usually found (figure R8) (**B**). In **B** we have outlined the domain border to better observe the spatial relationship between the basally extruding apoptotic cells (**B'**) and the superimposed apical cells with high levels of Yki (**B''**). Scale bar in **A** stands for **A** and **B**, while that in **A'** stands for the remaining panels.

It was important at this point to know if there were physiological consequences of the raise of these metabolic markers in the surrounding cells. To address this question we checked if the cell proliferation rate was affected in wild type cells next to the tumour border. In figure R11A we show a wild type disc cultured in a Bromo-2-deoxy Uridine (BrdU) solution. This molecule is incorporated in cells that are in the S phase of the cycle (see Materials and Methods for further details). Proliferation is homogeneously distributed over the disc. Interestingly, in the experimental discs there was a zone of increased proliferation localized at the border of the overgrowing domain (figure R11B'). This area partially overlaps with the band of apoptotic cells at the tumour border. A close up view of a domain border is shown in figure R11D. There it can be observed that dividing cells are present near dying cells. These extra divisions are found in the periphery of the tumour, most of them associated to non-GFP cells, as shown in figure

R11C, in which we show a single Z-section of a disc with a white line surrounding the tumour cells.

To analyse these tumour borders we generated their orthogonal section (figure R12). Thanks to this method, we saw that the tumours tend to be expanded in the basal region of the disc (bottom and right of the orthogonal views) and the dividing cells tend to appear superimposed apical to the dying cells (top and left of the sections). This was expected, as dying cells are normally extruded basally while nuclear division occurs on the apical side of the tissue.

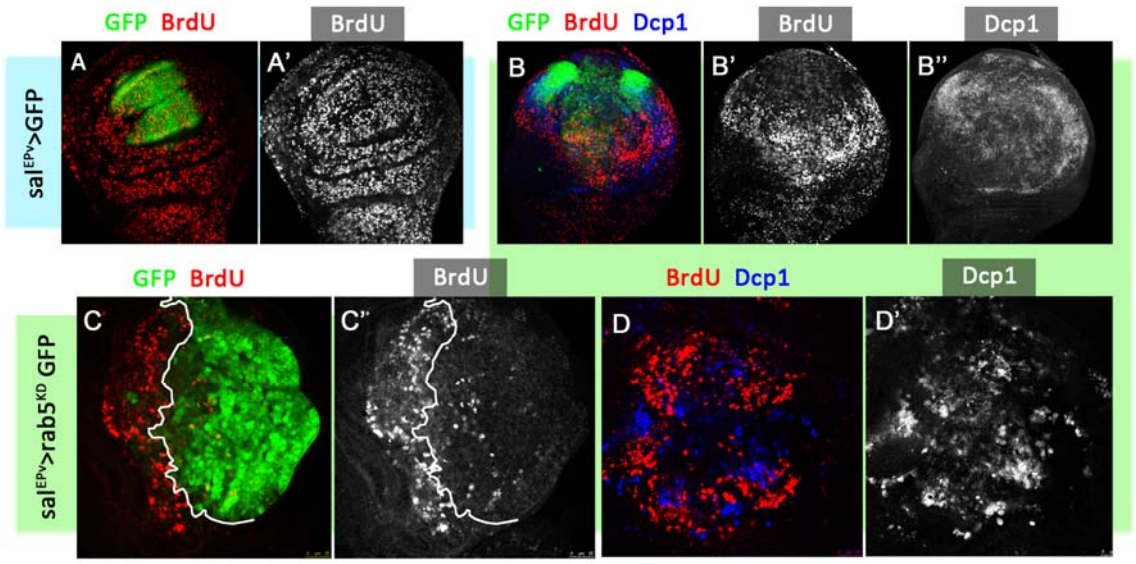


Figure R11. There is extra proliferation of cells next to dying tumour cells at the border of the domain. The distribution of dividing cells is homogeneous in GFP-expressing control discs as observed by BrdU incorporation (**A**). Experimental discs, however, show an increased proliferation rate in cells surrounding the *sal*^{EPv} domain (**B-D**). A co-stain of Dcp1 and BrdU reveals a spatial association of apoptotic and dividing cells (**B, D**). **B** is a high magnification of a region of a domain border. Dcp1 marked cells belong in all cases to the domain.

Similar results to those just described were obtained by using an anti-phospho-Histone 3 (PH3) antibody, which detects cells entering mitosis.

Together, these results point to a possible effect of the dying tumour cells on the non-tumour ones, increasing their metabolism, fitness and proliferation rate.

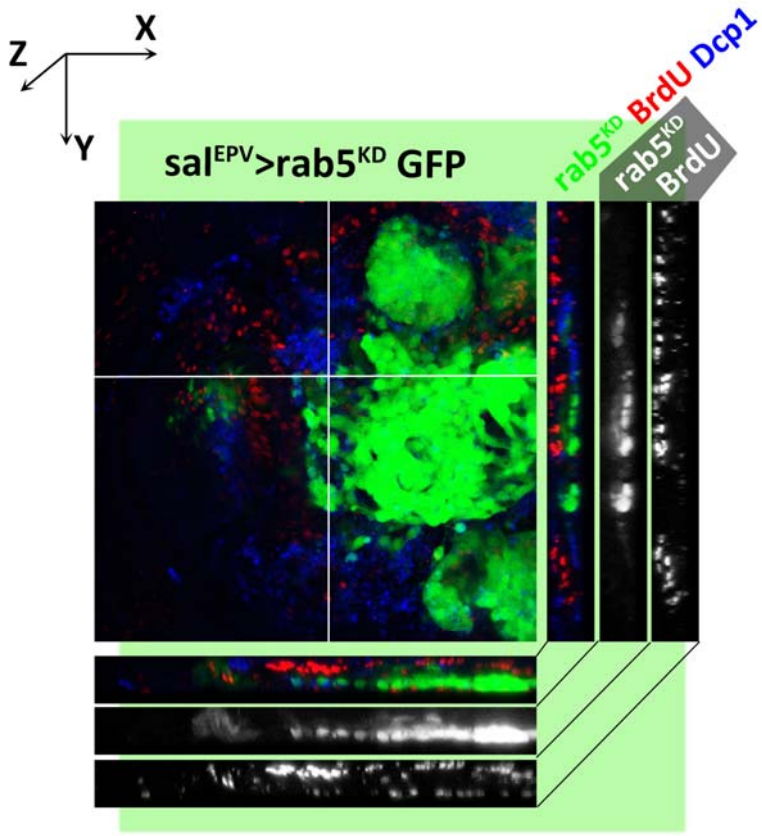


Figure R12. Orthogonal section of a *sal^{EPv} rab5^{KD}* tumour border. An orthogonal section of a whole-disc stack is shown. It was performed where the white lines cross the image. Basal region of the disc corresponds to the bottom of the X-section and to the right of the Y-section.

Wild type cells are recruited into the tumour

If cells at the border of the tumour are eliminated by cell competition and surrounding cells respond by having extra divisions, one possibility that we wanted to test was whether these cells occupy the space left by the dying tumour cells. In other words, we investigated if there was cell recruitment into the tumour.

As a first approach we traced the lineage of the *sal^{EPv}* domain. To achieve this we forced the expression of the recombinase *Flippase* (*Flp*), with a UAS-*Flp* construct, under the transcriptional control of the *sal^{EPv}*-Gal4 driver, temporally modulated by Gal80^{TS}. A lineage-marking cassette was inserted as well, *ubi FRT (stop) FRT GFP*. A cell with this genotype that lies at the *sal^{EPv}* domain at the permissive temperature (29°C) will express *Flp*, which will in turn induce the cis-recombination between the FRT sequences, resulting in the expression of GFP (*ubi-GFP*) (see Materials and Methods). Any cell that belonged to the *sal^{EPv}* domain at any time during the induction period at 29°C will therefore get permanently marked by GFP. A replica of these experiments was performed with a lineage-tracing cassette that marked the cells by the expression of *lacZ*, and similar results were obtained (not shown).

In control discs all the cells in the *sal*^{EPv} domain were marked by GFP. Random cells located at different sites in the disc were found also to be marked, but this was not reproducible and happened in a negligible number of cases. We can conclude that this lineage-tracing reproduced the *sal*^{EPv}-Gal4 expression pattern (figure R13A).

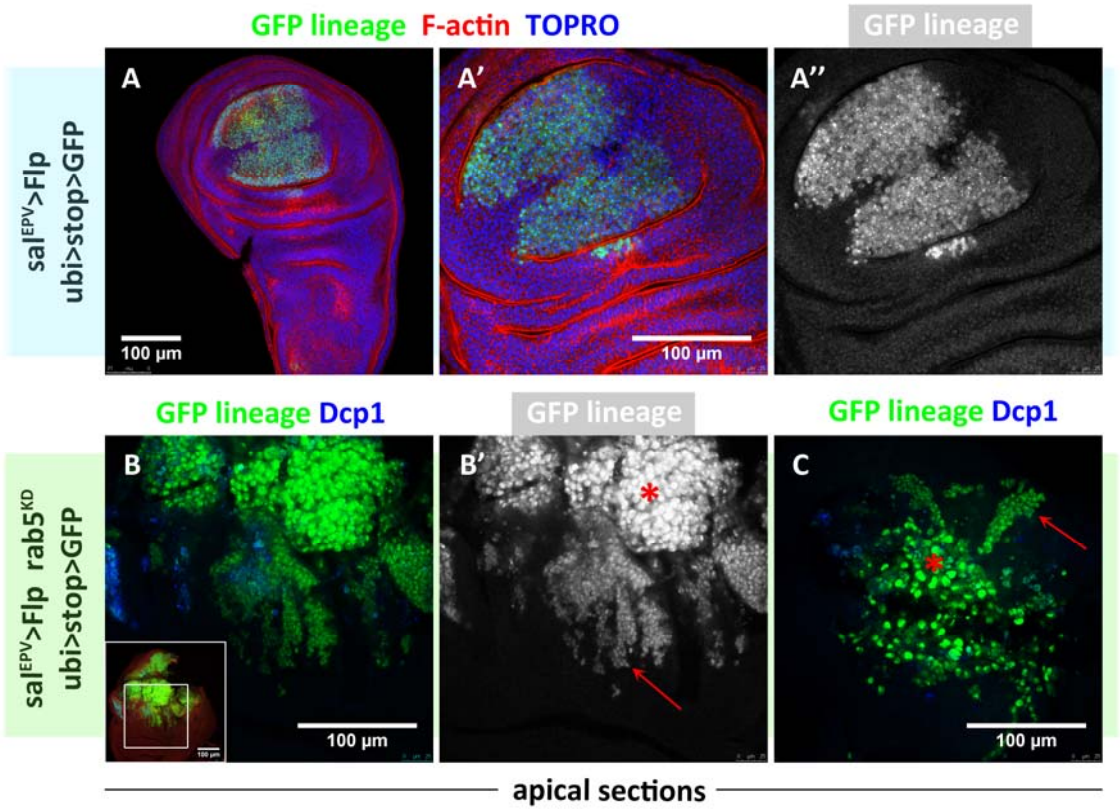


Figure R13. Lineage tracing suggests active cell recruitment into the tumour. Cell lineage is efficiently marked by expressing *UAS-flp* under the control of the *sal*^{EPv} driver, which induces the cis-recombination of a cassette that marks permanently the cells by expressing *GFP* under the promoter of *ubiquitin* (**A**). **B-C** Experimental discs in which *rab5* function is impaired in the domain while marking its lineage overgrow to the same extent as previous cases (**B, inset**). A close-up view of the apical surface of the tumours reveals the existence of two visually distinguishable cell populations (asterisks and arrows in **B'** and **C**).

We then induced the *rab5*^{KD} tumour in the *sal*^{EPv} domain while simultaneously marking the cell lineage of the *Gal4* line. This method allows us to follow the evolution of the group of cells that belonged to the tumour. We induced tumour formation and lineage marking for three days (29°C). After this time, larvae were let to recover at 17°C for one day and then analysed the imaginal discs. Interestingly, we could distinguish two cell populations marked by GFP (figure R13B-C). The most abundant one had the traits of the neoplastic tumours, multi-layered and disorganized tissue, with round-shaped cells and some dying cells at the border (asterisks in B'

and C). The less abundant, GFP marked, cell type was located in the apical sections of the disc and had a wild type shape, size and organization (arrows in B' and C). They were always found at the periphery of the tumour-like marked cells. No Dcp1 activity was detected in this apical population.

We interpret these results as a visualization of an apical recruitment of wild type cells that become marked by GFP as soon as they enter the sal^{EPv} domain but are not transformed into tumorous ones due to the switch to 17°C by the time they were acquiring the sal^{EPv} identity.

This would be a dynamic phenomenon in which cells at the periphery of the tumour are being eliminated by cell competition while wild type cells adjacent to the border of the tumour respond with extra proliferation and get recruited to it. Once there, they would activate their *sal^{EPv}-Gal4* driver, expressing, as a consequence, GFP and $rab5^{KD}$ therefore becoming transformed, contributing to the growth of the tumour.

Due to the dynamic nature of this phenomenon, the best method to confirm our hypothesis was to study the behaviour of the tumour border along time. We could achieve this by using a powerful confocal microscope that allowed us to take thousands of pictures of cultured living discs without affecting its development. Nuclei were marked by the *histone-2-RFP* transgene and tumours were induced by expressing $rab5^{KD}$ under the control of sal^{EPv}–Gal4 and $Gal80^{TS}$ and marked by GFP. Gal4 expression was induced 24, 48 and 72 hours prior to dissection, *ex vivo* culture and imaging. At early stages (24 hours) we could detect apoptotic cells by the blebbing nuclei phenotype randomly distributed in the domain, which is consistent with the results shown in figure R8B. At this stage we did not detect tumour growth, cell movements or cell recruitment. However, 48 and 72 hours after tumour initiation these phenomena were indeed detected. In figure R14 we show selected time points of two movies, while the original video files can be found in the CD that accompanies this book (Movies 1 and 2). Both movies are the result of imaging two 48 hour-tumours for four hours at 29°C. It can be observed that the size of the GFP domain increases very rapidly. Movie #1 is a view from the apical side of a 3D-reconstructed disc. In the time-lapse images of figure R14 we point out the region of interest, in which several nuclei of wild type (non-GFP) cells sitting next to the tumour border acquire the GFP expression. We also show this analysis from Movie #2, which is the view of a subapical Z-plane of the disc, and the two orthogonal sections crossing the region of interest. In the region of interest of the X-Z section (on the right of the movie snapshots) it can be observed how the tumour cells are located more basally (basal to the left) while wild type

nuclei are entering the tumour apically (right). Along the movie these wild type cells acquire the tumour identity and the GFP marker.

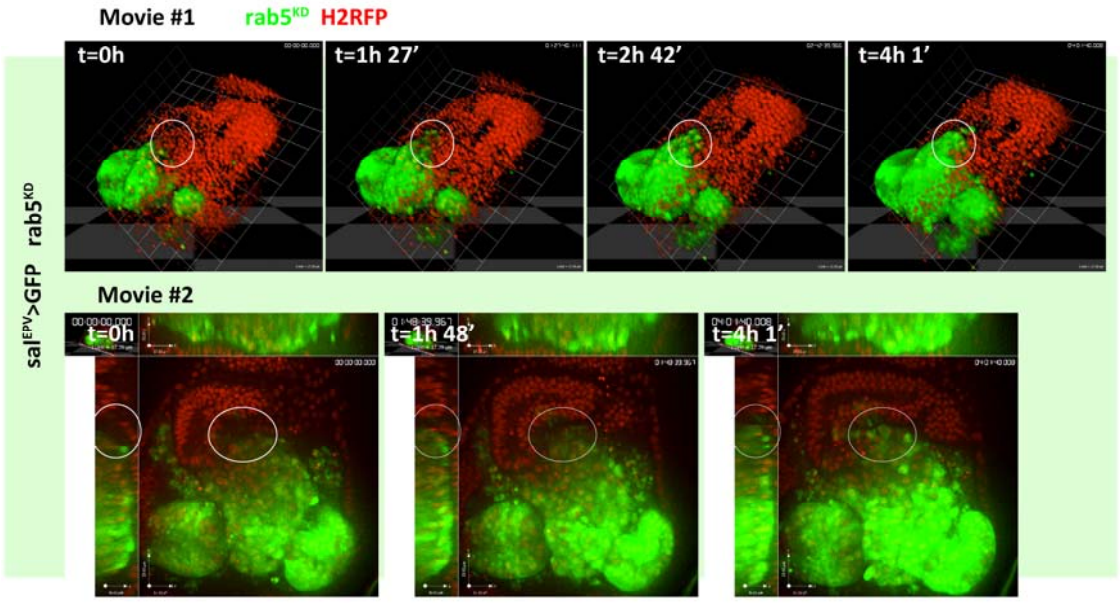


Figure R14. *ex vivo* imaging of growing tumours proves the acquisition of tumour identity by wild type surrounding cells. Snapshots of two four-hour movies are shown with significant steps of the progression of *sal*^{EPv} *rab5*^{KD} tumours. Nuclei are marked with Histone 2-RFP and tumour cells with GFP. **Movie #1** is a 3D reconstruction of a whole-disc stack movie and is here presented from the apical view. **Movie #2** is the view of a single Z-plane at the subapical level of the tissue. In both analyses the acquisition of GFP by non-tumour cells can be observed within the circled regions.

This material was further studied to understand if there was a spatiotemporal relationship of blebbing (dying) and dividing cells. We made a systematic analysis of the timing of these events and in several Z-planes. As mentioned before, most apoptotic cell bodies are found basally while adjacent mitotic nuclei can be found mostly apically. For this reason, to analyse all the events occurring during a movie we divided the disc in four sections of similar thickness from apical to basal and merged the Z-planes that composed each section. By doing this, we obtained four movies containing the information of the original one divided in four Z-sections. In this way we could observe an apoptotic event occurring in the basal movie and analyse if there are consequences in the apical movie. However, to establish a temporal relation, we divided each movie into four one-hour movies. There we could already detect all the events happening in each section and search for consequences in the other sections later on (see analysis method in Appendix Figure 1).

The results, presented in Appendix Figure 2, are schematic views of an outlined *sal*^{EPv}-Gal4 *rab5*^{KD} domain with apoptotic events coloured in purple and mitotic nuclei in yellow. We found several foci of dying cells that could be related to more apical mitotic events happening either within the same hour or at later times. Two of these foci are indicated in the figure. Circled in red there is an apoptotic focus that along time gets extruded basally while in the second and third hour of movie we can observe mitotic events apical to the apoptotic cells (red arrows). Similarly, there is another region with apoptotic cells (green circle) that shows a proliferative hub apical to it (green arrows). Although these observations do not imply a cause-consequence relationship between dying and dividing cells they suggest that there could be a spatiotemporal connection that is further investigated in forthcoming experiments.

Altogether, the experiments described in this section led to the conclusion that wild type cells respond to the presence of the tumour with extra divisions and are recruited to it.

JNK pathway is responsible for the mitogenic signalling

We know from the clonal analysis of *rab5*^{KD} that these cells activate the JNK pathway, which induces the expression of different genes with a wide range of functions during development and disease (reviewed in (Rios-Barrera and Riesgo-Escovar, 2012)). Also, it has been shown that JNK, under certain circumstances, induce the expression of the morphogens Wg and Dpp. These have been proposed to be involved in the induction of extra proliferation (Huh et al., 2004; Perez-Garijo et al., 2004; Perez-Garijo et al., 2009; Ryoo et al., 2004). These results suggested that JNK could be activated in the *sal*^{EPv}-Gal4 *rab5*^{KD} tumours and involved in the emission of mitogenic signals. Therefore, we checked on the expression of *puc-lacZ* as an indicator of JNK function. As expected, we found that JNK was highly activated throughout the domain, as compared with control discs in which *puc-lacZ* is active only in the most proximal tip of the disc, the so-called stalk (figure R15A). After a detailed examination of all the cases, we conclude that only the GFP-marked cells (tumour cells) or apoptotic debris are marked by the presence of *puc-lacZ* (figure R15B, C). To double check that the pathway was active we stained against the product of one JNK target gene, *matrix metalloproteinase-1* (*mmp1*). Similar results were obtained (figure R15E, F).

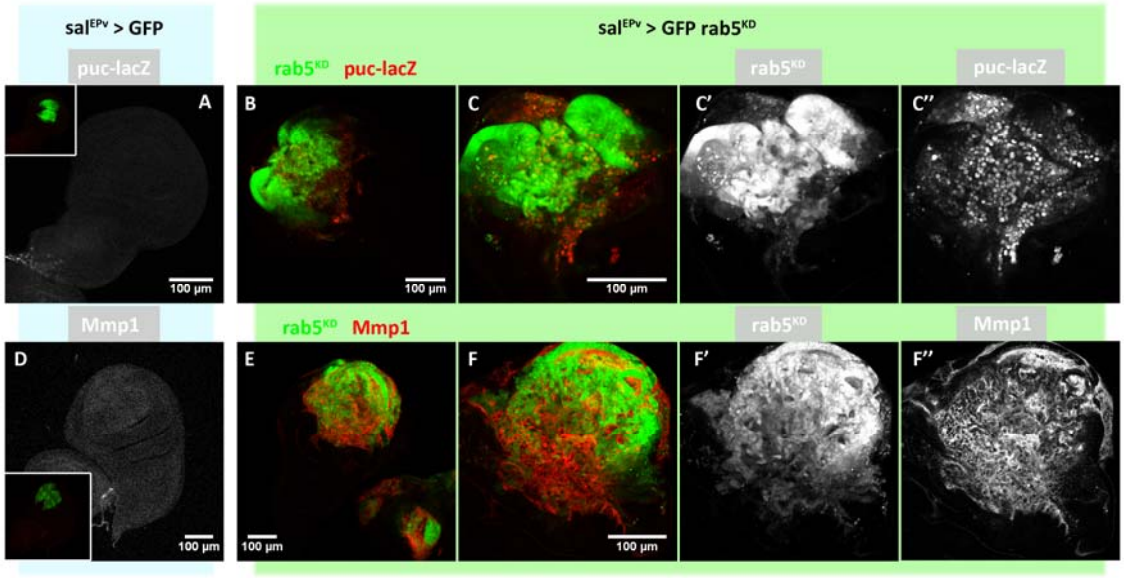


Figure R15. JNK is activated in *sal^{EPv} rab5^{KD}* tumours. Control discs show a restricted expression of the JNK reporter *puc-lacZ* to the stalk region (**A**). Upon tumour induction in the *sal^{EPv}* domain there is an increase of *puc-lacZ* signal, indicating an activation of the JNK pathway (**B-C**). Mmp1 levels were also checked to assess the activation of JNK through the increased expression of a target gene. While Mmp1 is not found in wild type discs (**D**), a clear up-regulation is observed specifically in tumour cells (**E-F**).

Once we had proved that JNK is highly active in these tumours, we investigated whether there was also an associated expression of *wg* or *dpp*. For this purpose, we used a Wg antibody and a *dpp* RNA probe. Interestingly, the tumours had a massive upregulation and mislocalization of Wg compared to the wild type discs (figure R16A, C). *dpp* mRNA was slightly increased within the domain as well. This, together with the JNK pathway activation, supported the hypothesis that wild type cells could be responding to these signals which had been linked to extra proliferation. However, a functional analysis is needed to prove the requirement of JNK for the extra proliferation of wild type cells.

As a functional assay we tested three genetic contexts that abolish apoptosis or JNK activity. First, we expressed *rab5^{KD}* in the *sal^{EPv}* domain together with a double stranded RNA against the gene encoding the apical caspase Dronc (heretofore named *dronc^{KD}*). By this method we were interfering with the apoptotic pathway only in the tumour cells, while the rest of the disc remains wild type. Apoptosis was very effectively eliminated (figure R17A'). Interestingly, this produces a drastic reduction of the size of the domain (figure R17D), which nevertheless did retain some tumorous features. The tissue was irregular and the cells had aberrant shapes. Together with the suppression of the tumorous overgrowth, we also found that the intense proliferation of the tumour border was reverted to a normal rate (figure R17A'').

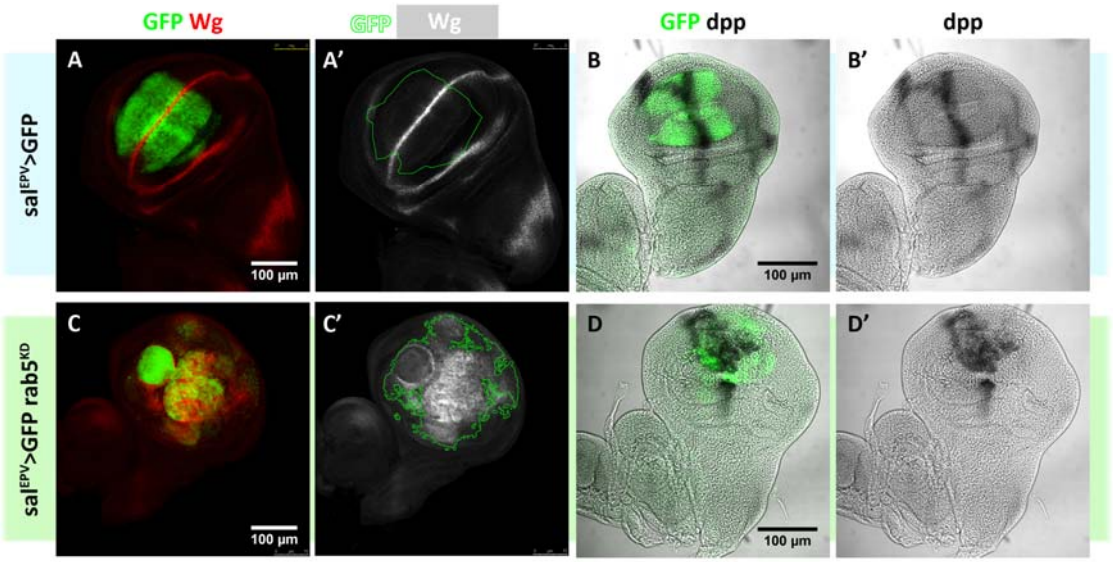


Figure R16. *wg* and *dpp* are upregulated in *rab5*^{KD} tumours. We present in **A** and **B** control discs with normal Wg protein distribution (**A**) and *dpp* mRNA expression (**B**). We induced tumour transformation with *rab5*^{KD} under the *sal*^{EPv} driver control in **C** and **D**. Wg protein is highly overrepresented in experimental discs and localized throughout the tumour domain (**C'**). Tumour domain has been outlined in **C'** to better distinguish the area of interest. Similarly, *dpp* is overexpressed in this context as observed by *in situ* hybridization of an mRNA probe (**D'**).

To avoid having two interference RNAs, which could saturate the processing machinery, and also to reduce the number of UAS sequences, which could titrate the amount of Gal4 protein available, we also generated *sal*^{EPv}*>rab5*^{KD} tumours in a *dronc* mutant background. This prevents caspase activation in the entire organism, not only in the tumour domain as in the case previously described. As it is presented in figure R17B, D, very similar results are obtained. We find that the tumour size is much reduced and there are low apoptotic levels. The proliferation rate surrounding the domain is also normalized. However, the tissue architecture does not revert to wild type, maintaining some minimal folds and cell shape aberrations.

The third set of experiments was designed to block JNK activity within the tumours, which can induce the ectopic expression of *wg* and *dpp*. We overexpressed (together with *rab5*^{KD}) the gene *puckered* (*puc*), which is a negative regulator of the pathway, with the UAS-*puc*^{14C} transgene. Not only did the JNK inhibition lead to low levels of apoptosis and to a normal proliferation rate around the domain, but it also restored much of the tissue architecture and the size and shape of the domain (figure R17C-D).

All the preceding experiments indicate that cell death within the tumour is necessary both for the increased proliferation and the tumour overgrowth. Moreover, JNK appears to be

upstream the apoptotic program and may also be responsible for the oncogenic traits of *rab5*^{KD} cells.

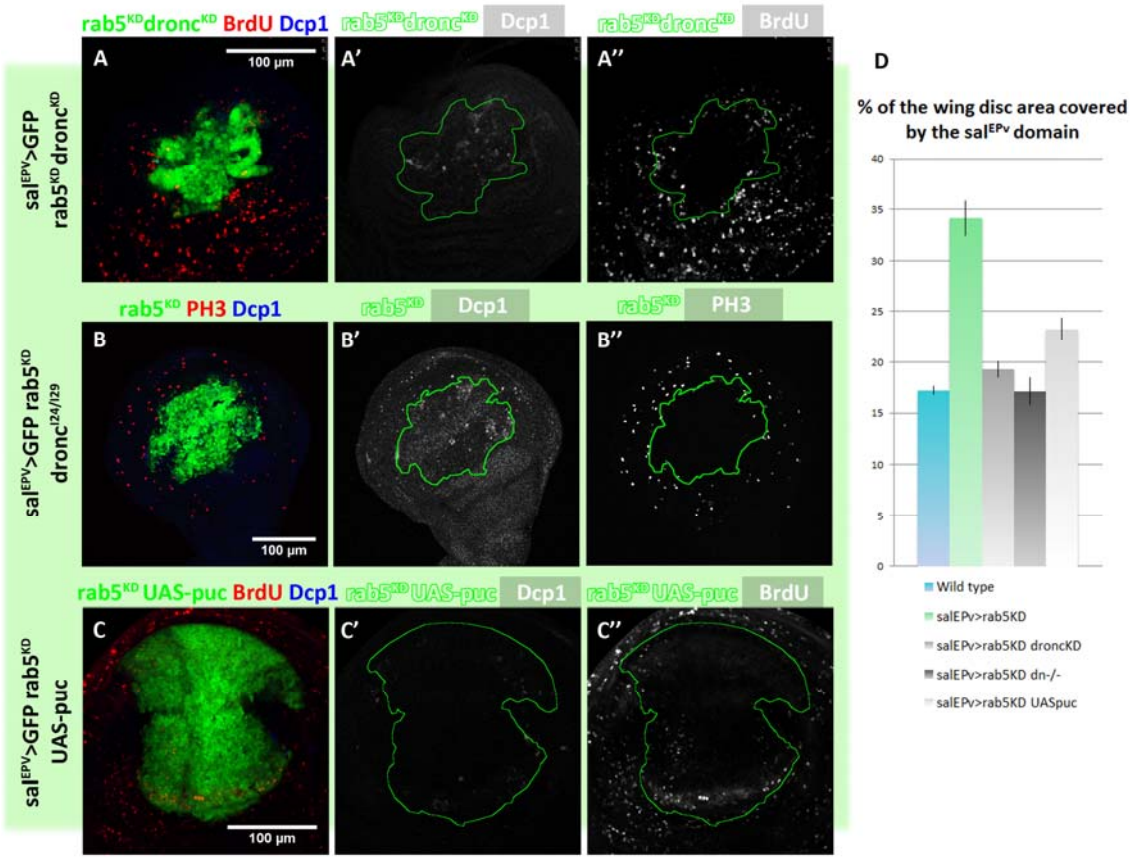


Figure R17. Tumour overgrowth and associated proliferation depend on JNK and apoptosis. We induced *rab5*^{KD} expression in the *Sal*^{EPv} domain in the absence of apoptosis. To block apoptosis we used two different methods: expression in the domain of an interference RNA against the apical caspase *dronc* (*droncKD*, **A**), or a mutant background for *dronc* using two alleles (*droncI24* and *droncI29*, **B**). To inhibit the JNK pathway we overexpressed in the domain the JNK inhibitor *puckered* (*UAS-puc*, **C**). In all three genetic contexts apoptosis was efficiently eliminated as seen by the absence of Dcp1 (**A'**, **B'** and **C'**). We did not find excessive proliferation at the domain boundary (**A''**, **B''** and **C''**) as seen by the presence of PH3 or BrdU. We quantified the size of the domain with respect to the total disc area (**D**).

An important control was to demonstrate that in these experiments, in which the absence of *dronc* activity prevents the *sal*^{EPv}*>rab5*^{KD} domain from developing into an overgrowing tumour, the cells are still deficient in *rab5* activity. To this effect we blocked apoptosis by using the *dronc*^{KD} transgene. In the *sal*^{EPv}*>rab5*^{KD} *dronc*^{KD} experiment *we* incubated the experimental and control discs in comparable Dextran solutions and observed that it was highly accumulated in the *rab5*^{KD} domains independent of the genetic context (figure R18), meaning that endocytosis was blocked in all the experiments presented before.

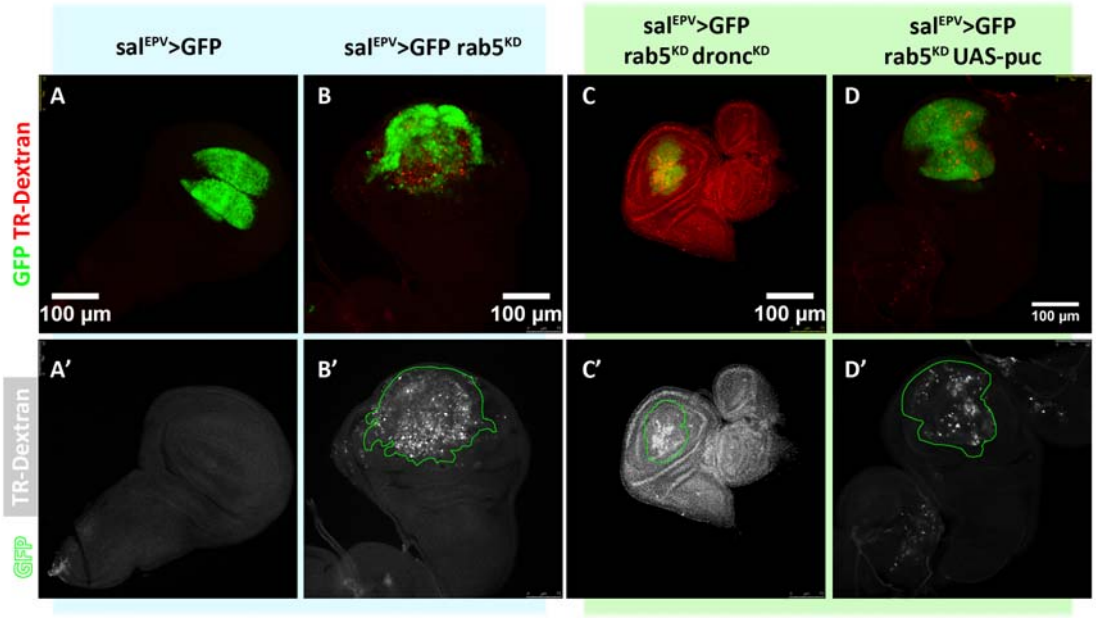


Figure R18. Blockage of apoptosis or JNK pathway does not affect the endocytic defect of *rab5^{KD}* cells. We incubated control and experimental discs in comparable Texas Red-Dextran solutions to check on the endocytic efficiency of the tissue in each context. Wild type control discs completely endocytosed the fluorescently marked sugar (**A**). As previously reported, *rab5* deficient cells are unable to process endocytic vesicles and therefore accumulate Dextran on their surfaces (**B**). The same phenotype of lack of endocytosis was obtained when apoptosis or JNK were impaired (**C** and **D** respectively).

Still, the ectopic expression of *wg* and *dpp* in the *rab5^{KD}* tumours could be an autonomous misregulation due to the lack of endocytosis in the cell and not due to cell death or to JNK activation. We therefore analysed their expression when apoptosis or JNK are genetically abolished. In the first case, in which we express *rab5^{KD}* and *dronc^{KD}* under the control of the *sal^{EPv}* driver, we can observe a strong rescue in the pattern and localization of the Wg protein, as observed in figure R19A, and compared to the control and *rab5^{KD}* experimental case in figure R16. The *dpp* mRNA expression band in the disc is also mostly restricted to the wild type pattern (figure R19B).

Similarly, when blocking the JNK pathway in the *sal^{EPv}>rab5^{KD}* wing discs by expressing UAS-*puc^{14C}*, both *wg* and *dpp* expression levels and pattern are almost indistinguishable from the wild type (figure R19C-D, compare with figure R16).

These results indicate that JNK would be responsible for cell death, mitogenic signalling, tumour growth and several tumorous features of the *rab5^{KD}* cells.

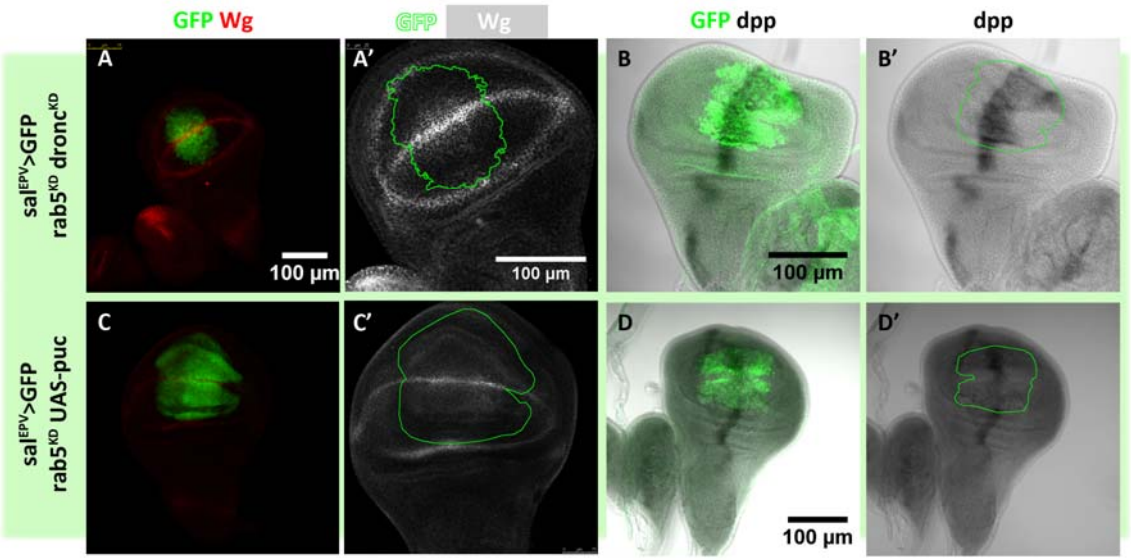


Figure R19. Wg and *dpp* pattern are restored when apoptosis or JNK are abolished. *sal*^{EPv} domains deficient for *rab5* in which apoptosis (**A-B**) or JNK activity (**C-D**) are blocked show an almost wild type pattern of Wingless (**A'**, **C'**) and expression of *dpp* (mRNA) (**B'**, **D'**). In panels **A'**, **B'**, **C'** and **D'** the domain has been outlined in green to facilitate the observation of the region of interest. Scale bar in **A** stands for **A** and **C**, and the one in **A'** stands for **C'** too.

Altogether, in this section we have presented evidence indicating that the apoptosis generated downstream cell competition stimulates tumour growth. More concretely, outcompeted cells activate autonomously the JNK pathway, which in turn leads to *wg* and *dpp* ectopic expression. These mitogenic signals are received by cells close to the tumour border. Although some *rab5*^{KD} cells may receive these signals and induce some extra proliferation, our results show that the group of cells that respond most significantly to *wg* and *dpp* are the wild type cells surrounding the tumour. They increase their metabolic status and their proliferation rate. Moreover, they enter the overgrowing domain and acquire tumorous features, thus contributing to further growth of the tumour.

Mechanism of Wg by which it induces extra proliferation

The role and signalling mechanism of Wingless and Dpp to induce proliferation in an otherwise wild type disc remains controversial in the field. They have been described as morphogens that are expressed along the compartment boundaries during disc development: *dpp* is expressed in a stripe of anterior cells along the Anterior/Posterior (A/P) border while *wg* is expressed at the Dorsal/Ventral (D/V) boundary. From their expression domains a protein gradient is created. This spread generates a subset of domains of expression of several downstream

target genes that respond to Wg or Dpp in a concentration-dependent manner, and these, in turn, are responsible for the correct patterning of the adult structures. However, their direct involvement in cell proliferation is not clear yet. Genetic manipulation of their pathways triggers both autonomous and non-autonomous responses in terms of cell cycle arrest or promotion that vary depending on the particular cells that receive them (Diaz-Benjumea, 1995; Johnston and Sanders, 2003) (Capdevila and Guerrero, 1994). These morphogens are not required to compensate disc size with extra cell proliferation after a massive cell loss in the wing imaginal disc, suggesting that other mechanisms may govern this response (Perez-Garijo et al., 2009). Interestingly, very recent work proves that Wg spread is dispensable for tissue growth and proliferation during normal development (Alexandre et al., 2014). Altogether these arguments lead to the idea that Dpp and Wg might only induce or block cell proliferation when misexpressed.

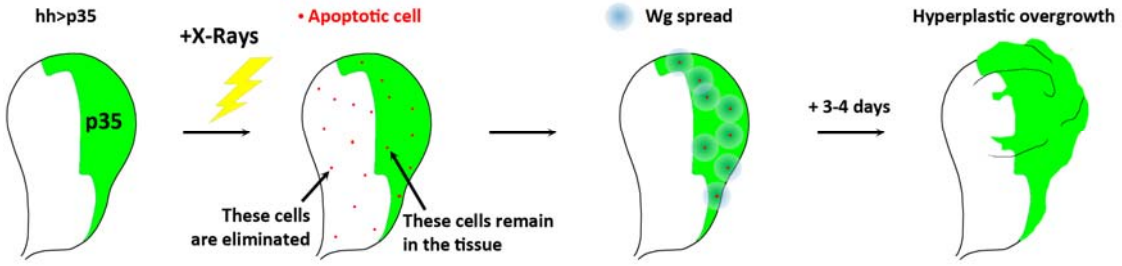


Figure R20. Schematic model of the generation of undead cells in the wing disc. Here we show a model that represents the basic features and steps in the generation and development of undead cells. Apoptosis is blocked by the expression of *p35* under the *hedgehog* promoter (*hh*), which drives the expression to the posterior compartment (P), marked by green colour (representing GFP). Apoptosis is induced in the entire organism by irradiating it with X-rays. Apoptotic cells appear throughout the tissue, although only the ones in P remain. These start secreting mitogenic signals, such as Wingless (Wg), which spread and induce extra proliferation of cells at a distance.

In our tumour model we have shown that the ectopic expression of *wg* and *dpp* downstream of JNK is correlated with extra proliferation of receiving cells at the vicinity. This suggests that these signals could be spreading and triggering proliferation in nearby cells. To test this hypothesis we used a model of *undead cells*, which are known to activate ectopic *wg* and *dpp* and induce extra cell divisions in their surroundings (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004). This system consists of inducing apoptosis in a tissue that expresses the baculovirus protein p-35, which blocks the activity of effector caspases. This results in a tissue with random cells that initiated apoptosis but are unable to die, so they stay in the tissue in an indefinite apoptotic state, permanently releasing Wg and Dpp (see schematic model in figure

R20). Here we present experiments we have performed to reveal the role and signalling mechanism of Wg in this context.

Wingless spread contributes to the extra proliferation associated to the undead cells

To generate undead cells, we expressed *UAS-p35* under the control of the *hh*-Gal4 driver, which is specific of the posterior compartment, and induced apoptosis by treating the larvae with a high dose of X-rays as previously described (Perez-Garijo et al., 2004). Non-irradiated control discs had a normal behaviour and development. No phenotypes have been associated to the sole expression of *p35* and normal adult organisms are recovered. However, in the experimental irradiated larvae hyperplastic overgrowths can be observed affecting to the posterior compartment of the imaginal disc. Associated to these overgrowths there is an increase in the mitotic rate, as evidenced by the PH3 staining, which is higher in the posterior compartment and in the anterior cells in contact with the A/P border (figure R21B, arrow). Similar results are obtained with the incorporation of BrdU (not shown). As it was also previously described (Perez-Garijo et al., 2004; Ryoo et al., 2004), we observed groups of cells in the P compartment with ectopic Wg expression (figure R21B'). Interestingly, we often find tissue folding surrounding or associated to the groups of *wg*-expressing cells, which could be a sign of a mitogenic role of Wg. These overgrowths also generate indentation that disturb the normally straight line separating the A and P compartments. It has been hypothesized that JNK may have a role in this phenomenon due to its ability to promote cell migration.

To test whether Wg spread had a relevant role in triggering extra proliferation, we induced undead cells in a genetic background in which the *wingless* gene is fused to *neurotactin* (*NRT-wg*). This transgene was described elsewhere (Zecca et al., 1996) as a membrane-tethered non-diffusible form of Wg. Interestingly, we found that the induction of undead cells in this context did not induce extra proliferation or overgrowths to the same extent as in a wild type background (figure R21C''). The extra proliferation found previously in the anterior cells in contact with the posterior compartment is not observed in the experimental background either. To quantify the size difference, we measured the proportion of the disc covered by the posterior, GFP marked, compartment in our experiment and in two different controls: irradiated discs in which the P compartment expresses *p35*, and irradiated discs of NRT-wg heterozygote larvae, which do not express *p35* (figure R21D). It can be observed in the graph that in all time points the *NRT-wg* homozygosis prevents the hyperplastic overgrowth reaching similar values to the control discs. This observation supports the idea that undead cells

produce ectopic Wg that in normal circumstances spreads to reach further cells and induces extra proliferation in them.

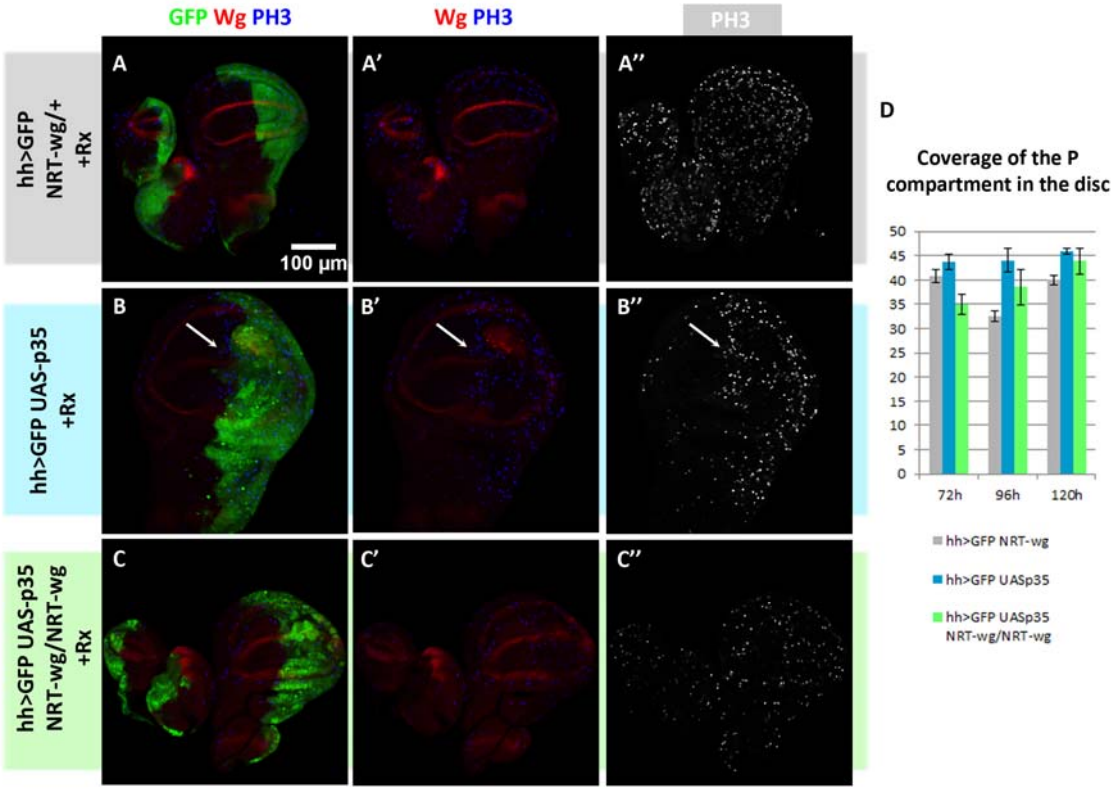


Figure R21. Increase of cell proliferation and compartment size is correlated with Wg spread. All samples in panels A-C were irradiated with a 1500R dose and dissected 48h after irradiation. Disc of wild type phenotype that expresses *GFP* in the P compartment and is heterozygous for *NRT-wg* (**A**). It shows a wild type Wingless pattern (**A-A'**) and normally distributed proliferation, visualized with PH3 antibody (**A''**). If *p35* is expressed in this compartment it induces ectopic foci of *wg*-expressing cells (**B-B'**) and extra proliferation localized to the P compartment (**B''**) and a band of cells in the A region closest to P (**arrow**). If, in this scenario, Wingless does not spread (working in a *NRT-wg* homozygous background), we do not find ectopic Wingless foci (**C-C'**) and proliferating cells' distribution is mostly restored (**C''**). The excess of proliferation in P is a sign of the excess of growth observed upon quantification at different time points (**D**). Discs with undead cells show overgrown P compartments (**B, D blue**) as compared to wild type discs (**A, D grey**). However, if Wg spread is prevented, the size of the P compartments is partially rescued (**C, D green**).

wingless ectopic expression in undead cells depends on Wingless spread

Along the course of these experiments we made an observation that may be relevant to the field of cell signalling and cancer. *wg* expression pattern changes along the development of the wing disc. In early stages it has a broad expression throughout the presumptive wing blade. During the third larval stage, at the end of the wing disc development, its expression becomes restricted to two rings surrounding the presumptive wing blade, the D/V boundary within the

wing pouch and a band of cells at the presumptive notum region (see figure R21A). When undead cells are induced in a wild type *wg* background, others and we have shown that there are groups of cells that express *wg* ectopically – *i.e. wg* acquisition in cells located away from its normal domain of expression (figure R22A'', asterisks). However, if they are induced in a *NRT-wg* background the protein pattern changes drastically.

In figure R22 we show representative cases of discs in which undead cells have been induced in the posterior compartment (*hh*-Gal4, *UAS-p35*) by X-irradiation. We imaged the entire discs in the Z-axis using a confocal microscope and projected the stack. This analysis allowed us to observe in one image all the events happening in the apical, medial and basal regions of the tissue. Interestingly, the stain with anti-Wg antibody reveals that in the posterior compartment of *NRT-wg* homozygous discs, the pattern of Wg is irregular as compared to control discs, although very rarely a Wg positive cell can be found detached from the normal domain of expression (figure R22B'', asterisks).

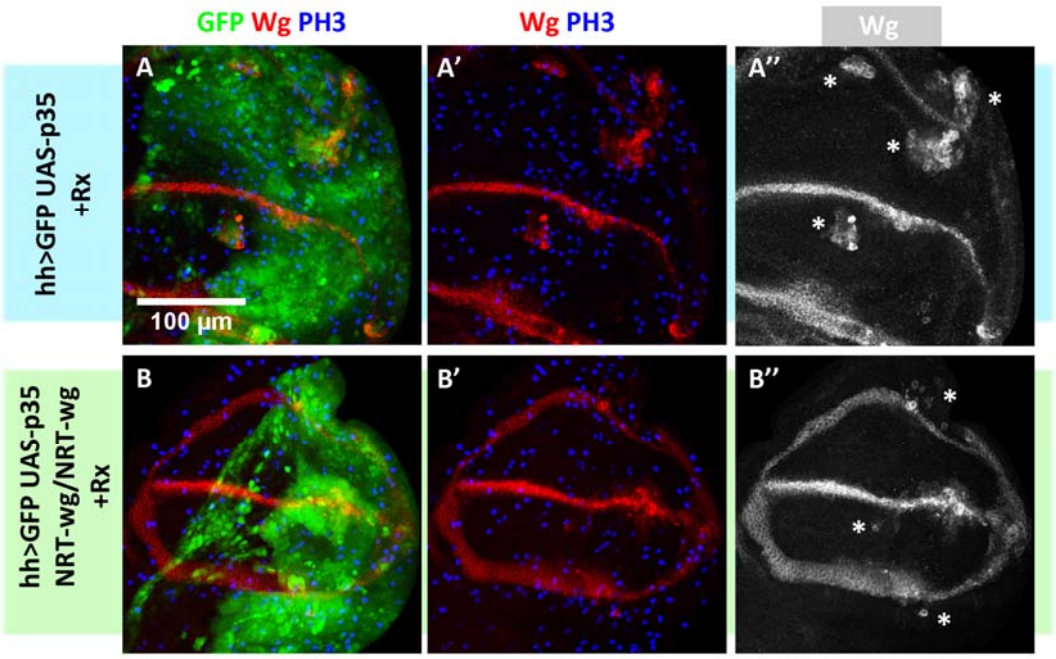


Figure R22. Wingless spread induces a change in Wg pattern and a decrease of extra proliferation. To better visualize the effects of eliminating Wg spread, we show a maximal projection of all the Z-planes of discs of the indicated genotypes (**left**). When there is wild type *wg*, we find ectopic expression of it in undead cells (**A''**) and excess of proliferation associated to it (**A'**). If Wg spread is prevented, the ectopic expression of it is restricted to discrete cells (highlighted with **asterisks**) neighbouring the domain of expression of the gene at this stage (**B''**). Again, the distribution and amount of PH3-positive, proliferating cells is mostly normal (**B'**).

The mechanism by which Wg spread would induce *wg* expression elsewhere is yet to be elucidated. However, we can claim that abolishing its spread, the amount and location of *wg*-expressing cells is reduced in the tissue and this correlates with a lower rate of proliferation of cells at the vicinity.

We have proved in this section that not only Wg spread can induce extra proliferation in surrounding cells but also it induces foci of distant cells that express it. The ability of wild type Wg to induce extra proliferation is therefore multiplied assuming that the ectopically expressed *wg* plays the same mitogenic role.

Search for upstream regulators of cell competition

Cell competition is nowadays an active field of study in *Drosophila* and in vertebrates due to its implication in both cancer suppression and progression. Much effort has been put in defining different models and mutations that, when in a mosaic tissue, can trigger this process. A number of mechanisms have been suggested to be involved in the elimination of cells during cell competition, such as activation of the JNK pathway, phagocytosis or the circulating haemocytes. However, there is less unanimity when it comes to defining the upstream trigger for cell competition, as the elements proposed do not apply to all cell competition models (see Introduction for further details). This led us to the hypothesis that there may not be a unique upstream regulator in all the cases of cell competition. Something that seems to be accepted is that the element(s) must act between cells in close contact, probably through a relative comparison of some trait of the confronted cells.

In our laboratory we have worked extensively with several tumour suppressor mutations that cause mutant cells to generate neoplastic overgrowths in a homotypic environment but be eliminated by cell competition in a mosaic tissue. These oncogenic cells present important cytological differences when compared to wild type cells, and we propose that these differences are detected and as a consequence cell competition occurs.

We designed a method to screen for upstream modulators of cell competition. Candidate genes were selected following two different criteria (see below). To test their involvement in the process we induced mutant clones for a tumour suppressor gene, which are normally eliminated when confronted to wild type cells, and expressed a double-stranded RNA (dsRNA) against each of the candidate genes in the clones.

The tumour suppressor gene that we used for these experiments was *lethal giant larvae* (*lgl*), which has been studied and characterized in depth in our laboratory and others (Agrawal et al., 1995; Bilder et al., 2000; Gateff, 1978; Menendez et al., 2010). Lgl, together with Scribbled and Discs large, belongs to a protein complex in the basolateral region of epithelial cells that regulate the appropriate apico-basal polarity of the cells by interacting with the Par and the Crumbs complexes. The loss of cell polarity involves changes in cell shape, cell adhesion to neighbour cells and to the substrate, location of transmembrane proteins and protein recycling. We hypothesize that these cell surface differences with respect to wildtype cells could be sufficient to initiate a cell competition interaction. Therefore, our first criterion to select candidate genes was their involvement in processes that could be mediating the *lgl* characteristic surface phenotypes (transmembrane proteins, cell-cell adhesion proteins…) or their ability to receive juxtacrine signals (membrane receptors).

As a second criterion we selected genes that are accessible for the transcription machinery in a context of cell competition when compared to controls, which could be interpreted as genes that might be specifically expressed upon the interaction of cells that leads to cell competition. This genomic information was obtained from a member of the laboratory, who conducted a Whole-Genome Chromatin ImmunoPrecipitation sequencing (ChIP-seq) analysis in which he screened the genome for dimethylations in the lysine 9 of the Histone-3 (H3K9 diMet) or for its acetylation (H3K9 Ac), which are traits of transcription silencing (heterochromatin) or activation (euchromatin), respectively (unpublished data). Three different tissues were assayed: wild type and $lgl^{4/4}$ mutant wing imaginal discs as controls and lgl^{4} *UAS-ras*^{V12}/wild type mosaic discs, in which high levels of cell competition take place (Menendez et al., 2010). Using these data we selected genes that were highly acetylated in lgl^{4} *UAS-ras*^{V12} tissues as compared to lgl^{4} or wild type controls to find genes that are activated specifically upon cell competition and are not misregulated due to the *lgl* mutation itself. Complementary to this selection, we also searched for genes that were highly dimethylated in wild type tissues and also highly acetylated in lgl^{4} or lgl^{4} *UAS-ras*^{V12} discs. With this we selected genes that were expressed specifically in *lgl* cells, and therefore could tag them as losers when confronted to wild type cells.

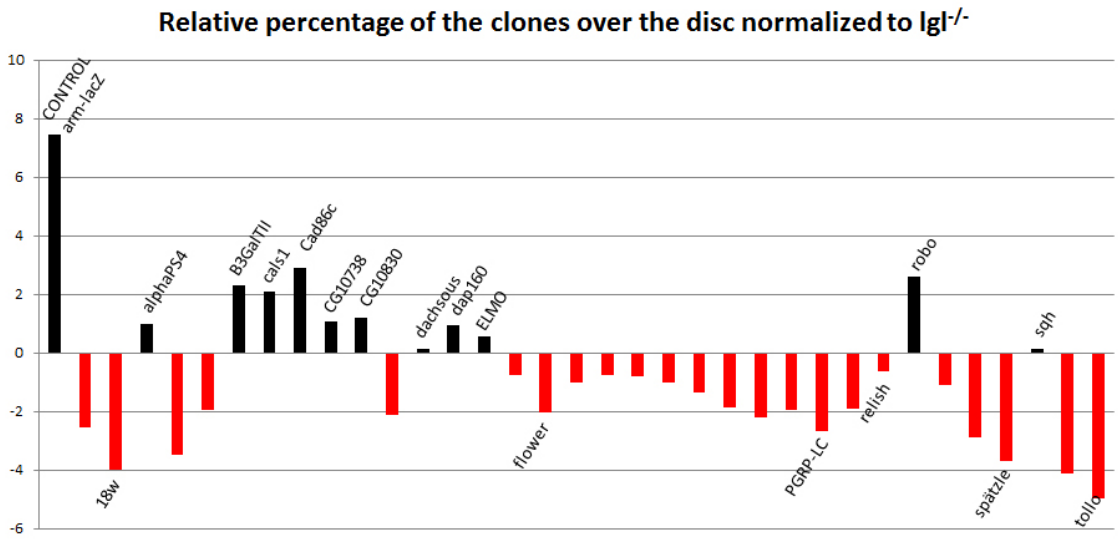


Figure R23. Effect of different dsRNAs on the elimination of *lgl* mutant clones. The disc occupation of *lgl*$^{-/-}$ clones was measured and compared to that of wild type *lac-Z*-expressing control clones and that of *lgl*$^{-/-}$ dsRNA clones. We normalized *lgl*$^{-/-}$ clones' disc occupancy to zero and represent in the graph the percentage of the disc that clones of each genotype cover relative to *lgl*$^{-/-}$. Black filling was given to positive values while negative values were coloured in black. We identify positive hits and those that are discussed further in the text. The full list of dsRNAs screened so far can be found in Appendix table 1.

A rough examination of the candidate genes led to the observation that the mitochondrial respiration machinery was specifically enriched in tissues with cell competition. Several elements of this system were tested.

The list of the candidate genes tested so far is presented in Appendix table 1, although this is an ongoing work and more candidates will be screened. The ability of the *lgl UAS-dsRNA* clones to survive within a wild type tissue was quantified and used as a measure of the possible involvement of the candidate genes in cell competition. We compared the size of these clones with *lgl* mutant control clones, which are mostly eliminated after 3-4 days after clone induction. As a second, positive, control we compared the experimental discs to *armadillo-lacZ*-expressing wild type clones growing within a wild type context. We measured the area covered by the clones in the wing discs, which correlates to the ability of the clones to survive cell competition. In figure R23 we show the graph we obtained after a normalization of the data for *lgl* mean coverage value. Therefore positive (black) values in the graph are considered hits of candidate genes that improve *lgl* clones' survival while negative (red) values are obtained when the candidate genes decrease their viability.

We know from previous work (Menendez et al., 2010) that *lgl* mutant cells, when not confronted to wild type cells, divide at approximately the same rate as wild type cells, although they do not stop dividing when the final organ size is reached. Therefore, if *lgl* clones were rescued from cell competition, we would not expect to find severe overgrowths but close-to-normal sized clones by the end of larval development. This means that a complete blockage of cell competition would lead to a disc' coverage of the clones of about 7-8% more than that of *lgl* mutant clones, comparable to the *armadillo-lacZ* wild type clones (figure R23). However, none of the genetic combinations assayed reached those values. Among the positive hits there are several genes related to heterophylic or homophylic cell-cell interactions, most of them cadherins, integrins or immunoglobulin-like transmembrane proteins. This supports our hypothesis that cell interaction is needed for cell competition to occur and that transmembrane proteins should be mediating it.

Interestingly, some of our candidate genes have been related to cell competition by other authors. On the one hand the expression of the dsRNA against *flower* (*fwe*) rescues wild type loser clones in a background with high dMyc activity (Rhiner et al., 2010). We used the same dsRNA used by the authors and this resulted in a decrease of viability of *lgl* clones (figure R23, *fwe* is pointed out among the negative results). It has been shown that *scribble* mutant clones activate two isoforms of *fwe* (fwe^{LoseA} and fwe^{LoseB}), which drives their elimination through cell competition (Rhiner et al., 2010). We tested whether the $Fwe^{LoseA/B}$ isoforms were generated in our models of cell competition. We induced $rab5^{KD}$ clones in a wild type background that carried a *flower* transgene in which each isoform is tagged differentially (Yao et al., 2009). In this context we could only see the ubiquitous isoform and none of the Fwe^{Lose} isoforms. This, together with our finding that *fwe* dsRNA does not rescue lgl lethality, suggests that this mechanism might not take place in our cell competition models.

On the other hand it was recently proposed that cells may use a novel combination of elements of the Toll and Imd pathways to accomplish cell competition (Meyer et al., 2014). However, *lgl* mutant clones are not rescued when this pathway is interfered at several levels (see figure R23, elements indicated among the negative values).

Altogether, we have some evidence indicating that the currently proposed pathways to detect unwanted cells (*fwe* and Toll/Imd models) do not rescue *lgl* clones from cell competition. More experiments would be needed to finally discard these possibilities. Instead, we have found some cellular functions that could be involved in *lgl* detection and/or elimination. Future work could determine their particular role in cell competition.

DISCUSSION

Dual role of cell competition in tumorigenesis

During development cells may spontaneously acquire mutations that compromise the function of organs or the life of the organism. In *Drosophila* there is a surveillance mechanism, cell competition, that identifies and eliminates these aberrant or suboptimal cells. The salient features of cell competition have been described in recent reviews (Amoyel and Bach, 2014; Baillon and Basler, 2014). Recent work (Claveria et al., 2013; Sancho et al., 2013) has shown that cell competition is not a specialty of *Drosophila*, for it performs similar in mouse development.

Experiments done mostly in *Drosophila*, but also mammals have shown that cell competition is able to eliminate cells with wrong identity (Adachi-Yamada and O'Connor, 2002; Milan et al., 2002), decreased metabolism (Bohni et al., 1999; Johnston et al., 1999) or a low proliferation rate (Morata and Ripoll, 1975; Oliver et al., 2004) as compared to their neighbouring tissue.

Interestingly, there are several reports pointing to an anti-tumour role of cell competition, as it can detect and specifically eliminate oncogenic cells (Chen et al., 2010; Grzeschik et al., 2010; Igaki et al., 2009; Menendez et al., 2010; Tamori et al., 2010). Another case was recently reported that points to a tumour-suppressive role of cell competition. Normal function of mouse thymus requires cell competition to replace resident T lymphocyte progenitors by incoming progenitors from bone marrow. If cell competition is prevented, resident progenitors develop a T-cell acute lymphoblastic leukaemia (T-ALL) (Martins et al., 2014).

In this work we have provided further evidence supporting this anti-tumour role of cell competition. However, we have also described how, under certain circumstances, it can play an unexpected tumour promoting function and propose a mechanism underlying this phenomenon. We have tested a particular mitogenic signal (Wingless) that could be involved

in such tumorigenic response. Finally we have shown a preliminary work that aims to identify upstream modulators of cell competition in charge of the detection of tumour cells.

Cell competition eliminates oncogenic *rab5*-deficient cells

The model we have used for this study responds to the basic definition of cell competition. Here we review the observations that led us to conclude that it is a valid model to study this phenomenon.

1. – The elimination of *rab5* mutant cells is context dependent. To eliminate the function of the small GTPase *rab5* we used a dsRNA (*rab5*^{KD}). Rab5 regulates a series of syntaxins that permit the fusion of endocytosed vesicles into the early endosome, a station from which cargoes are recycled back to the plasma membrane or sent for degradation in the lysosomes.

rab5^{KD} was expressed in the entire posterior (P) compartment to test the effects of this loss of function in a large region (figure R2). In these conditions we find that there is a massive overgrowth of the P compartment. The quantification method underestimates the actual growth because it does not consider the 3-dimensional volume of the compartment, which increases significantly upon *rab5* loss. This experiment also shows that *rab5*^{KD} cells are in general viable in those conditions. However, we find cells entering apoptosis, likely due to the inability of *rab5*^{KD} to complete the endocytic process. It is possible that JNK activity, which is known to occur autonomously in *rab5*-deficient cells (Takino et al., 2014), could be inducing an immune response of the *Drosophila* haemocytes and adipocytes, which, in turn, would drive apoptosis in tumour cells (Parisi et al., 2014; Pastor-Pareja et al., 2008).

Not only *rab5* has been described as a tumour suppressor in the literature. Mutant animals for most of the genes encoding for proteins of the endocytic machinery show neoplastic overgrowths of the imaginal tissue and nervous system (Lu and Bilder, 2005; Thompson et al., 2005). The induction of these tumours could be due to the accumulation of actively signalling molecules in the endocytosed vesicles (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005) or to the loss of apico-basal polarity, reported to occur upon endocytosis blockage and associated to tumorigenesis (Humbert et al., 2008; Lu and Bilder, 2005). In any case, cells with impaired endocytic machinery have been proved to be viable and form an organism.

However, although viable, *rab5*^{KD} or *rab5*^{2} (null mutant) cells growing in a wild type tissue are eliminated (figures R1 and R3). This indicates that *rab5*-deficient cells are selectively killed in a context-dependent manner, a characteristic of cell competition (Morata and Ripoll, 1975). The

activation of JNK in *rab5*^{KD} cells is another trait of cell competition (Moreno et al., 2002) (figures R1 and R3). The JNK pathway can play several roles (induction of motility, basal membrane degradation, cell death) in tumorigenesis. In our hands, *rab5* mutant clones do not metastasize or delaminate through the basal membrane (although there is Matrix Metalloproteinase secretion), probably due to their rapid elimination.

2. – The timing of elimination of *rab5* clones matches with that described in other cell competition models. We checked for cell death markers at different time points after clone induction and concluded that apoptosis started 24-48h after clone induction. Therefore, the timing of clone elimination is consistent with the idea that cell competition, which is known to happen after 24-48h of clone induction (Moreno et al., 2002), is responsible for the elimination of the *rab5*-defective cells.

3. – Apoptosis is mostly found at the interface between *rab5* and wild type cells, but not at compartment boundaries. It is accepted that cell competition is a short-range phenomenon that occurs upon interaction of two types of cells. If cell competition is responsible for the elimination of *rab5*-defective cells, cell death should be found in *rab5*^{KD} cells in contact with wild type cells. In cases like *rab5*^{KD} *Ras*^{V12} clones that are able to proliferate before elimination, it can be seen that dying *rab5*^{KD} *Ras*^{V12} cells localise at the periphery of the clone, just abutting normal cells (figure R4F). In the experiments in which we express *rab5*^{KD} in the *sal*^{EPv} domain we find an early pattern of apoptosis homogeneously distributed in the domain that could be attributed to autonomous death of *rab5*-defective cells. After 24-48h of *rab5*^{KD} expression we find a halo of cells with apoptotic markers similar to what we find in *rab5*^{KD} *Ras*^{V12} clones.

Moreover, if *rab5*^{KD} is expressed in the whole P compartment, we do not find apoptosis associated to the compartment boundary, the interface between *rab5*^{KD} and wild type cells. The compartment boundary is a characteristic barrier for cell competition due to an increased tension that avoids cellular intercalation (Levayer et al., 2015; Simpson and Morata, 1981).

A refinement of the microenvironment model

As described above cells defective in *lgl*, *scrib* or *rab5* function are eliminated by cell competition, even if they possess proliferative advantage provided by constitutive activity of the Ras pathway (Brumby and Richardson, 2003; Menendez et al., 2010; Pagliarini and Xu, 2003; Takino et al., 2014) or Notch (Brumby and Richardson, 2003). However, when there are numerous clones in the disc, these are able to form overgrowing tumours (Menendez et al., 2010). These results led to the *microenvironment hypothesis* (Menendez et al., 2010).

Taking that cell competition is a short-range interaction, only the cells at the periphery of mutant clones are eliminated. If a clone is too small, it has a high proportion of cells directly exposed to cell competition and therefore the cell elimination rate is probably higher than the proliferation rate of the inner cells; the clone disappears. In a big patch of mutant cells growing within a wild type tissue those in the periphery undergo cell competition, but the majority of those inside are beyond the reach of cell competition and can continue proliferating (depicted in figure D1). In this context, the rate of cell removal would be lower than the proliferation rate and therefore the tumour grows. The acquisition of a proliferative and survival advantage by expression of Ras^{V12} facilitates the growth and confluence of mutant clones in a tissue, therefore increasing their chance to overcome cell competition and generate overgrowths.

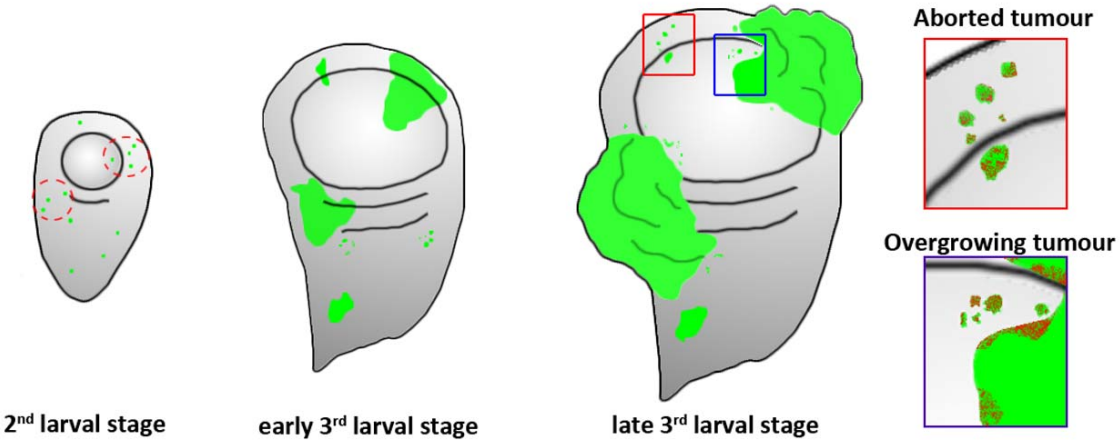


Figure D1. Two possible fates of oncogenic clones subject to cell competition. The microenvironment model. If many clones mutant for a tumour suppressor gene are induced in a disc, clone merging is facilitated (dashed red circle). Small patches (red square) have a high proportion of their cells exposed to cell competition and are rapidly eliminated. However, bigger patches (blue square) resulting from clone merging, have a small proportion of cells exposed to cell competition, thus permitting the proliferation of inner cells.

In this work we have presented several experiments that support this hypothesis and provide an estimate of the minimum size a patch must reach to overgrow.

1. – Merged *rab5*^{KD} *Ras*^{V12} clones can overgrow in a wild type tissue. We have shown that the tumorigenic potential of *rab5*^{KD} is comparable to that of *lgl* or *scrib* mutants when co-expressed with *Ras*^{V12}. As predicted by the microenvironment hypothesis, small clones tend to be eliminated due to the high proportion of clone cells exposed to wild type cells (figure R4F,

arrowhead), while in bigger clones or merged patches this proportion is lower (figure R4F, arrow) and tend to overgrow (figure D1).

The probability of merging is increased by generating a sufficient number of clones (about 23 clones per disc). When analysing the discs at increasing time points after clone induction we observe that the number of isolated patches of *rab5^{KD} Ras^{V12}* cells decreases (figure R5), indicating that the clones are either eliminated or merged. During this time we observe that the patches tend to occupy the entire disc, which becomes disorganized and unstructured (figure R5). We assume that in this case some clones are eliminated but clone merging may account for tumorigenesis because we rarely find tumours if few clones are induced.

2. – A group of approximately 400 cells is sufficient to generate a protective microenvironment. In this work and in previous reports the oncogenic potential of cells mutant for tumour suppressor genes was studied in conditions in which the discs were saturated with clones with a proliferative advantage and, therefore, merging was facilitated (Brumby and Richardson, 2003; Menendez et al., 2010; Pagliarini and Xu, 2003). Here we aimed to determine if the protective microenvironment could be obtained without the proliferative advantage (*Ras^{V12}*), and which was the minimum size of such patch of deficient cells.

We expressed *rab5^{KD}* in different domains of different sizes and positions in the wing imaginal disc (figure R7). The smallest domain that gave rise to robust overgrowths was that of the prospective wing blade, determined by the expression of the *sal^{EPv}-Gal4* driver (figure R7D and figure R8). We have calculated the approximate number of cells that constitute this domain by the time we induce the expression of *rab5^{KD}*. Taking that a mature disc contains about 31,000 cells (M. and Schneiderman, 1977; Martin et al., 2009) and that the *sal^{EPv}* domain covers about 16% of it (figure R8E WT), we conclude that it is composed of approximately 5,000 cells by the end of larval development. In most of our experiments, we induce the expression of *rab5^{KD}* at the end of the second larval stage. It is known that from that time the wing disc cells divide an average of 3.5 times before pupariation (Martin et al., 2009). Altogether, by the time of the temperature shift (*rab5^{KD}* expression), the *sal^{EPv}* domain should cover about 415 cells in a square-like shape. This size and shape leads to a scenario in which about 80 cells (20% of the domain) are exposed to cell competition, while the rest (80%) are beyond its reach, and therefore protected within the microenvironment.

We believe these results provide strong support for the microenvironment hypothesis and also suggest an explanation for the apparently conflicting behaviour of clones of oncogenic mutations.

Cell competition as a tumour-promoting factor. The *black hole* model.

Apoptosis in cancer research and clinical assays has long been considered as a mechanism to remove oncogenic cells having no further consequences. However, there is strong evidence showing that apoptotic cells are involved in signalling activities before dying. Experimental and clinical trials have proved that after irradiation treatments (or UV-radiation, heat or chemotherapy treatments), irradiated apoptotic cells can induce apoptosis non-autonomously in non-irradiated neighbours (reviewed in (Prise and O'Sullivan, 2009))(Perez-Garijo et al., 2013). This phenomenon is known as *bystander effect* and several pathways have been implicated in cell-cell signalling driving non-autonomous induction of cell death. It has been proposed that this spread of apoptosis can be of relevance to shape structures through collective cell death during development or to completely eliminate tumour cells that are not initially targeted by chemo- or radio-therapy (reviewed in (Prise and O'Sullivan, 2009)). How apoptosis is modulated to induce extra cell death in these contexts and not in all apoptotic events during the life of an organism has not been clarified yet.

Here we discuss how apoptosis derived from cell competition, which we and others have shown to have a tumour-suppressing function, can also in certain circumstances stimulate the growth of tumours. We also propose a mechanism of tumour growth linked to cell competition that may help understand certain clinical scenarios.

1. – Metabolic and proliferative response neighbouring apoptotic cells.

In our systematic analysis of *rab5*^{KD} tumours, we found that the region close to the tumour was enriched with cells with high levels of dMyc and Yki (figures R9 and R10). Yki functions as a transcription factor to trigger the expression of *inhibitor of apoptosis-1* (*dIAP1*), the cell cycle-promoter *cyclinE*, the micro-RNA *bantam* or *dMyc*. Altogether, the regulation of Yki coordinates tissue homeostasis. The activity of Yki and dMyc corresponds to a high metabolic status of the cells and a protection from apoptosis. Expectedly, this metabolic activation resulted in increased cell proliferation of the tissue close to the tumour (figure R11).

Both the increase of the metabolic and proliferation markers is found surrounding the border of the tumour, which corresponds to the zone of intense cell death due to cell competition. We could indeed find a spatial correlation of pools of apoptotic cells with neighbour cells that

accumulate dMyc (figure R9B''). Similarly, dividing cells often appear next to apoptotic cells in this region (figure R11D).

2. – Mitogenic signalling depends on apoptosis and JNK.

We have demonstrated that JNK is activated in *rab5*^{KD} cells targeted for elimination upon exposure to cell competition. Moreover, it has been described that endocytic blockage at the level of Rab5 induces an autonomous upregulation of JNK (Takino et al., 2014). In agreement with these observations, JNK is specifically upregulated in *rab5*^{KD} tumours at the *sal*^{EPv} domain (figure R15). However, it does not induce cell death in all the cells at the domain, which means that it could be playing a different role.

The *undead cells* model is a powerful tool to study the properties of cells in which the apoptotic pathway is activated, but that are unable to accomplish apoptosis through the effector caspases (for further details see *Undead cells* in the Introduction section). In this system it was observed that dying cells produce and spread mitogenic cues (Wg and Dpp) through the activation of JNK (Perez-Garijo et al., 2004; Ryoo et al., 2004). We therefore confirmed that such signalling was also found in our model (figure R16). The similarities between the undead cells model and our system pointed to the conclusion that JNK activity in the tumour cells could drive Wg and Dpp signalling, and these, in turn, could be the mitogenic cues that account for the ectopic proliferation surrounding the tumour.

Interestingly, functional analyses in our system proved the hierarchical relation of JNK and apoptosis as a trigger for *wg* and *dpp* ectopic expression and the subsequent extra proliferation associated to *rab5* tumours (figures R17 and R19). If apoptosis or the JNK pathway are inhibited in the tumour cells, *wg* and *dpp* show a wild type pattern, indicating that these signals are produced by apoptotic cells. Dying cells at the periphery of the domain only appear after 24-48h of *rab5*^{KD} expression (figure R8). This halo of apoptosis mostly replaces the homogeneous apoptosis pattern that appears early after *rab5*^{KD} expression. Assuming that it is the result of cell competition, we can suggest that at 24-48h of endocytosis blockage and at later times, cell competition-derived apoptosis is responsible for these mitogenic signals and thus for the extra proliferation associated with the tumours. Moreover, JNK may have an independent role in the neoplastic transformation of cells since inhibition of this pathway not only blocks apoptosis, Wg/Dpp signalling and proliferation, but also the shape and size phenotype of cells and the structure organization of the tissue (figures R17C, R18D, R19C-D).

In the literature there are a number of reports that address this proliferation associated to apoptotic cells. Concretely, the inhibition of the trafficking machinery in groups of cells has been linked to non-autonomous induction of proliferation. Examples of this are mutant cells for *erupted* (*TSG101*) or *Vps25*, two genes encoding for elements of the trafficking machinery. Mutant cells accumulate the intracellular domain of Notch in the vesicles with the subsequent production and release of Unpaired (Herz et al., 2006; Moberg et al., 2005). Unpaired acts as a ligand for the Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway in adjacent cells, stimulating cell proliferation (although it can also promote apoptosis). However, a cause-consequence relationship between apoptosis and signalling was not proved in these reports.

It has also been proposed that mutant clones for *rab5* induce non-autonomous proliferation through autonomous activation of Ras and JNK pathways that lead to the inhibition of the Hippo pathway, which would elicit the emission of Unpaired ligands (Takino et al., 2014). Importantly, in the same report, they prove that *rab5* mutation does not induce Notch accumulation, suggesting that Unpaired is produced downstream Yki. In this work the authors do not report whether apoptosis has a role in non-autonomous proliferation surrounding *rab5* clones. Actually, they test their hypothesis with *rab5* clones protected from cell death by the co-expression of *p35* or Ras^{V12}. It would be of great interest to check for an induction of *unpaired* in our tumour model in which protection from apoptosis (cell competition) is given by the formation of a microenvironment. We imagine that *unpaired* is not produced in our tumours due to its apparent dependence on Yki activity (Takino et al., 2014), which we do not find upregulated or localized to the nuclei within the tumour domain. Nevertheless, we have tested the possible role of Wingless in this non-autonomous proliferation downstream JNK, which is discussed below.

Another situation in which non-autonomous proliferation is observed takes place in the phenomenon of regeneration. In such case, increased proliferation is needed to recover a missing part of an organ or of the body. They all rely on an autonomous release of diffusible signals from apoptotic cells like the *Drosophila EGF* ortholog *spitz* (Fan et al.), the Wg ortholog Wnt3 (in *Hydra*) (Chera et al., 2009) or Wg (Smith-Bolton et al., 2009) among others (reviewed in (Vriz et al., 2014)).

All the mechanisms above proposed depend on the activation of JNK, which is consistent with our results. Mutant clones for several tumour suppressor genes are known to be eliminated through cell competition, and thus through activation of JNK (see *Cell competition* in the

Introduction section). We hypothesize that JNK activity in these clones could drive the emission of mitogenic factors that would account for extra cell divisions. However, they do not produce overgrowths since the mitogens' source cells and clones are rapidly removed from the tissue. This signalling downstream of JNK would explain the phenomenon of *interclonal cooperation* proposed by Wu *et al*. in 2010 (Wu et al., 2010) in which dying *scribbled* mutant clones would be a source of mitogens for the Ras^{V12} clones.

3. – Proposed model of tumour growth – the *black hole hypothesis*.

Among the results presented in this work there was one that specially caught our attention and interest. Not only did the inhibition of apoptosis or of the JNK pathway abolish the ectopic proliferation associated to the tumour boundaries, but it also eliminated the overgrowth of the sal^{EPv} domain. This suggested that apoptosis was the trigger for both the autonomous and the non-autonomous extra proliferation. However, we made some observations that proved that cells that originally did not belong to the tumour were recruited to it and acquired its identity. Therefore, the overgrowth of the domain has two components: the autonomous extra proliferation and the contribution of wild type cells that are integrated into the domain.

We describe using several approaches how wild type cells are recruited to the overgrowing domain.

- The systematic analysis of the 3-dimensional organization of the sal^{EPv} $rab5^{KD}$ domain revealed that while apoptotic cells at the tumour boundary were mostly extruded basally, non-GFP cells were superimposed to them apically (figure R12). This already suggested that wild type cells could be replacing those tumour cells that were eliminated.

- Further evidence came from lineage tracing experiments in which we induced in the sal^{EPv} domain the simultaneous expression of $rab5^{KD}$ and a *flippase*. The latter produces the recombination of a lineage-marking cassette which identifies cells from the domain, and their lineage, permanently. A temperature shift shuts down the expression of these transgenes and the evolution of cells that belonged to the domain (and their lineage) and that had tumourous traits can be followed. Interestingly we found a subset of marked cells located in the apical sections of the tumour border with no tumorous traits (figure R13B-C, arrow). These cells are distinguishable from the main tumour burden by the wild type size of their nuclei (*rab5*-deficient cells are known to have enlarged nuclei –figure R13B-C, asterisk–, maybe through endoreplication (Takino et al., 2014)) and by their organized growth in a unique cell layer. Our interpretation is that this subset of apical cells corresponds to cells originally external to the

domain but that substitute for tumour cells at the boundary. As they approach the domain they express the *Gal4* driver (*sal^{EPv}-Gal4* is a lineage-unrestricted domain of expression meaning that cells acquire or lose its expression as they approach or leave the central wing). The acquisition of this expression by previously wild type cells induces the cassette recombination and thus the permanent marking. However, these cells do not show a tumorous behaviour. We understand that more time of *Gal4* expression is needed to eliminate *rab5* function and exhibit its phenotype than the time needed to induce a recombination. Therefore, these cells would be those entering the domain after the temperature shift and are marked but not become tumorous.

- Lastly we confirmed the previous hypotheses through live-imaging the growth of the *sal^{EPv} rab5^{KD}* domain. Again, we observe that there is a dynamic replacement of dying cells at the tumour boundary by wild type cells that acquire the driver expression and contribute to the growth of the tumour.

We refer to this phenomenon as a *black hole* mechanism (depicted in figure D2) and we envisage it as follows: the growth of the tumour occurs by the continuous recruitment of non-tumour cells that fall into the *sal^{EPv}-Gal4* domain, where they become tumorous after they lose *rab5* activity. Many of the new tumour cells suffer cell competition, enter apoptosis, and secrete mitogenic signals that in turn induce proliferation of normal neighbours, some of which enter into the tumour and repeat the process.

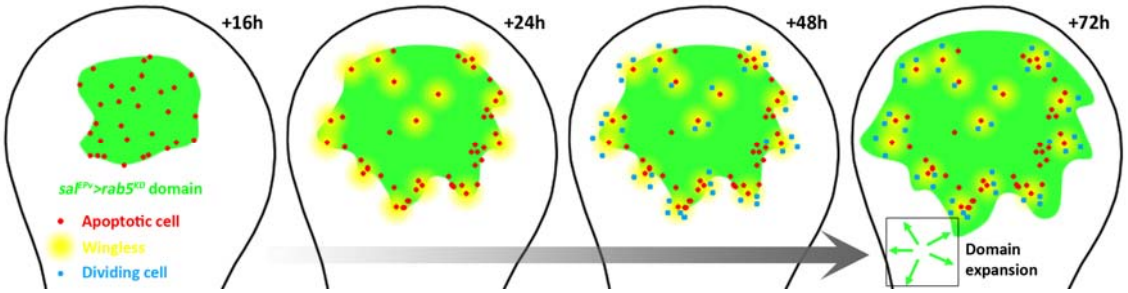


Figure D2. The black hole model. Here we depict the main steps of the evolution of a growing *sal^{EPv} rab5^{KD}* domain. Upon expression of *rab5^{KD}* we find a generalized pattern of apoptotic cells that in a few hours evolve and gets mostly restricted to the tumour boundary due to cell competition. JNK activity and cell death of tumour cells drive the ectopic expression of *wg* and *dpp*. The products are able to spread from the source cells and reach further non-tumour cells. These respond increasing their metabolism and having extra divisions. Some of these non-tumour cells at the boundary are recruited into the tumour and contributing to its further growth. We understand that once they acquire the tumour identity they are again exposed to cell competition starting the process again.

It is hard to extrapolate this kind of mechanism in clinical scenarios, but there might be some similarities in conditions like chronic ulcers or non-healing wounds, in which there are zones of persistent tissue damage and apoptosis that may be a continuous source of mitogenic signalling and could induce non-autonomous overgrowth of adjacent regions. Indeed, it has been reported that chronic ulcers triggered by infections of *Helicobacter pylori* can derive to gastric carcinoma (Parsonnet et al., 1991). The signalling mechanism we propose would account for an increase of proliferation associated to these lesions, although it would not explain a malignant transformation of the cells. This, in turn, could be achieved by the acquisition of other mutations taken that there are more divisions, and thus, higher risk of replication errors.

4. – Apoptosis-induced proliferation could be of clinical relevance.

It is generally accepted that apoptosis is an anti-tumour mechanism (Hanahan and Weinberg, 2011). However, we and others have provided evidence suggesting that apoptosis may also have a pro-tumoral role. There are a number of reports that warn that a high apoptotic index (AI) in human cancer samples is correlated with a poor prognosis of the disease (Jalalinadoushan et al., 2004; Leoncini et al., 1993; Nakopoulou et al., 2001; Naresh et al., 2001; Ohbu et al., 1995; Sun et al., 2006). Generally, the authors assume that a tumour with high AI needs more rounds of replication to acquire the clinically detectable mass, which facilitates the accumulation of mutations that transform the tumour. We suggest that apoptotic cells in those tumours are not silent and secrete mitogenic signals that may stimulate the growth of the tumour. Thus strong pro-apoptotic treatments (X-radiation, chemotherapy) to cancer patients may be counterproductive. Actually, it has been reported that tumour mammalian cells after heavy irradiation secrete the growth factor PGE_2 in a Caspase-dependent manner. This signalling induces extra proliferation of non-irradiated cells (Huang et al., 2011) and helps the repopulation of the tumour. A very recent report shows that apoptosis acts as a powerful growth promoter in human samples of aggressive B cell lymphoma (Ford et al.). Suppression of apoptosis in these samples strongly suppressed tumour growth, angiogenesis and accumulation of tumour-associated macrophages.

Role of Wingless spread in tumour formation

In this work we conclude that the overexpression of *wg* and *dpp* downstream of JNK in sal^{EPv} $rab5^{KD}$ tumours is necessary to induce non-autonomous proliferation. It is also known that *wg* or *dpp* loss of function inhibits the non-autonomous induction of proliferation in undead cells

(Perez-Garijo et al., 2009). They are assumed to be interdependent, as lack of one of them abolishes the ectopic expression of the other in these cells.

In the case of *dpp* and *wg*, the mechanism by which their protein products act to either permit or instruct cell proliferation during development is not known. The key issue is that they have restricted expression patterns in the imaginal discs but their proteins spread and create a gradient from the source. Their absence inhibits tissue growth, thus pointing to a role in cell survival or proliferation. However, dividing cells are homogeneously distributed in the discs throughout development, not being associated to Wg and Dpp gradients (for a more complete analysis see the Introduction section).

It was generally accepted that Wg had to diffuse and reach distant cells to induce the expression of target genes involved in pattern formation during wing development (Zecca et al., 1996). However, a recent report has shown that flies carrying a non-diffusible form of Wg as the sole source of Wg (*wg* {KO; *NRT-Wg*}) display a normal wing pattern, suggesting that indeed Wg diffusion does not play a major role in this process (Alexandre et al., 2014).

In this context, we decided to test the role of Wg spread in the induction of proliferation after tumour induction. As said, the elimination of Wg, or of its signalling pathway, in a tissue during the generation of undead cells inhibits the extra proliferation, suggesting that Wg could act either permitting or instructing proliferation at the vicinity of undead cells. We have induced undead cells by X-irradiating larvae that express *UAS-p35* in the posterior compartment, under the control of the *hh-Gal4* driver, mimicking the conditions used in previous reports (Perez-Garijo et al., 2004; Perez-Garijo et al., 2009). Instead of abolishing Wg function throughout the compartment (with the subsequent developmental abnormalities) we used a homozygous background for the non-diffusible form of *wg* (*NRT-wg*).

The comparison between the behaviour of discs containing undead cells in a wild type *wg* background or in a *NRT-wg* background led to two significant observations.

1.- Wg acts at a distance inducing cell proliferation

We have found that wild type Wg induces a higher amount of dividing cells at the vicinity of undead cells than does the membrane-tethered Wg. This can be visualized in the non-autonomous effect across the compartment boundary. Wg emanating from posterior undead cells can induce proliferation in anterior cells only when it can spread (figure R21B, arrow). These results suggest that Wg protein must reach cells to induce extra cell division, having an instructive role in this pathological situation.

The increased proliferation induced by normal Wg results in the overgrowth of the compartment (figure R21D). However, we find that impeding Wg diffusion does not rescue the size of the P compartment to wild type values, but the overgrowths are modest.

The partial rescue of the proliferative and the overgrowth phenotypes in a *NRT-wg* background suggests that *dpp* could still be ectopically produced and account for the non-autonomous proliferation that remains. This is an ongoing project and we are working to test this hypothesis.

2.- Wg spread is involved in the induction of ectopic foci of Wg

Unexpectedly, we have observed that the amount and distribution of ectopic Wg upon generation of undead cells depends on Wg spread. As previously described, undead cells can express *wg* in regions of the disc where the gene is normally not transcribed. Although groups of ectopic *wg*-expressing cells tend to be located at the proximity of the *wg* domain of expression, they are not necessarily in contact with it. However, in a *NRT-wg* genetic background, these foci are composed of fewer cells that appear tightly connected to the domain of expression of *wg* (R22).

As the only difference between these experiments is the ability of Wg to spread, we assume that a possible role of undead cells-derived Wg is to diffuse and induce its own expression in distant cells. However, this is not likely to occur as there are several reports stating that *wg* overexpression does not lead to induction of *wg* in distant cells.

Thus we hypothesize that upon generation of undead cells (in a wild type *wg* background) Wg diffuses and reaches other groups of undead cells acting perhaps synergistically with JNK (which is active in these cells) to permit its own transcription. If Wg is unable to diffuse it would not reach further JNK-positive cells and we would not find ectopic expression of *wg*, consistent with our observations.

Finally, we cannot discard other pathways that could be implicated in the stimulation of *wg* expression. We have reviewed that under certain circumstances the JAK/STAT pathway is involved in the non-autonomous induction of proliferation (Herz et al., 2006; Moberg et al., 2005; Takino et al., 2014). It is also known that JNK can induce the expression of *unpaired-3*, which is an activating ligand of JAK/STAT (Kolahgar et al., 2015; Pastor-Pareja et al., 2008; Wu et al., 2010). We imagine that, upon diffusion, Wg could activate, or cooperate with JNK and activate the JAK/STAT pathway via Unpaired-3.

Possible role of Wg spread in other models of tumour development

Our results would indicate that during normal development *wg* expression and diffusion would induce overgrowths or extra foci of ectopic *wg*-expressing cells. Importantly, this is not the case. What we have proposed is that this novel mechanism driving the ectopic *wg* expression probably involves the JNK pathway which is inactive during normal development in the imaginal discs. JNK activity is crucial for undead cells-associated overgrowths (Perez-Garijo et al., 2009; Ryoo et al., 2004). Also, we have proved that tumour cells subject to cell competition activate JNK, which in turn leads to the ectopic expression of *wg* and *dpp*. Associated to these oncogenic cells, there is a massive proliferative wave in wild type tissue at the vicinity (figure D2). Whether Wg spread is responsible for this extra proliferation or for the widening of its domain of expression (through induction of its expression in other cells) is being currently addressed in the laboratory. Other tumour contexts are being taken into account as this extra growth mechanism could be a general issue of many cancer models, in which JNK is active and *wg* is misexpressed.

How are tumour cells detected prior to cell competition?

Several combinations of different interacting cell types are known to trigger cell competition. Here we have validated a new model to study it: *rab5*-deficient cells are eliminated by cell competition when confronted to a wild type cellular context. Loss of function of other tumour suppressor genes in discrete groups of cells has also been linked to the induction of cell competition (Brumby and Richardson, 2003; Grzeschik et al., 2010; Igaki et al., 2009; Menendez et al., 2010; Tamori et al., 2010). These cells have the potential to divide for an extended period to generate neoplastic tumours albeit with a normal proliferation rate (Menendez et al., 2010). Thus, cell competition triggered upon confrontation of these mutant cells with a wild type tissue is not due to differences in cell division rate, as suggested for other cell competition models (Morata and Ripoll, 1975; Oertel et al., 2006; Oliver et al., 2004; Rodrigues et al., 2012; Tyler et al., 2007).

Understanding what are the cues that drive the specific elimination of tumour cells is of great interest for cancer research. So far, there have been two attempts to uncover the mechanism of identification of unwanted cells by wild type ones (see “Mechanisms of cell competition” in the Introduction section), although they have not been proved to be involved in tumour models (Meyer et al., 2014; Rhiner et al., 2010). We have presented a preliminary screen to search for upstream modulators of cell competition in a tumour model (*lgl* mutant clones in a wild type context).

We have included in the screen members of the two machineries previously described to take place in other models:

First, *flower* (*fwe*) was described as a transmembrane protein with three isoforms that differ in their extracellular domain. Cells normally express a ubiquitous isoform (fwe^{ubi}) while loser cells expose the two other isoforms (fwe^{LoseA} and fwe^{LoseB}) (Portela et al., 2010; Rhiner et al., 2010). We expressed in the *lgl* mutant clones a dsRNA against all three isoforms and this potentiated the elimination of the clones. This result is consistent with the *flower code* proposed by the authors, in which the loser condition is acquired by the expression of the $Fwe^{LoseA/B}$ isoforms or by the downregulation of the Fwe^{ubi} isoform. If we are targeting with the *fwe* dsRNA all three isoforms within the *lgl* clones, these would have a decreased amount of the Fwe^{ubi} isoform, and are therefore highly eliminated. However, the authors are able to rescue loser clones with the same dsRNA line in a dMyc context of cell competition (Rhiner et al., 2010). This fact indicated that the *flower code* does not account for the detection of tumour cells. However, since there are other elements downstream *flower*, we plan to test their implication in the elimination of tumour cells in future work.

Second, it has been proved for two different models of cell competition that unwanted cells are detected and killed by a novel pathway that uses elements of the innate immune system of *Drosophila* (Meyer et al.). These elements were described as sensors of non-self virulence factors, such as yeast, bacteria or fungi, and mediate a humoral immune response against them. In cell competition they act in a different manner, activating the proapoptotic genes *hid* or *rpr* in the loser cells. We have shown that the blockage of this novel pathway at several levels did not rescue *lgl* clones' lethality, indicating that the detection and elimination of *lgl* cells may not use these elements.

Interestingly, the dsRNAs against several other candidate genes partially rescued *lgl* mutant clones, suggesting that they could play a role in their elimination. In accordance with the generally accepted idea that cell competition relies on direct interaction of confronting cells, we found among the candidates that rescued the viability of *lgl* clones several cadherins, integrins or immunoglobulin-like proteins. Interestingly, other unexpected functions that were not previously related to cell competition have appeared in this screen. For instance, protein glycosylation could be mediating *lgl* elimination. This is likely to be an interesting research line to be followed since it has been described that cancer cells acquire a non-self cellular surface identified by altered protein glycosylation. However, the immunogenicity of these alterations is still under study (reviewed in (Amon et al., 2014)).

CONCLUSIONES/CONCLUSIONS

Conclusiones

1.- Células tumorales mutantes para *rab5* activan la vía JNK y mueren por apoptosis si están en un contexto genético normal aunque son viables en un contexto homotípico. La competición celular tiene un papel supresor de tumores al eliminar estas células mutantes para *rab5*.

2.- Al dar una ventaja proliferativa a clones mutantes para *rab5* se facilita la fusión de clones y la formación de un microambiente protector.

3.- Un tamaño mínimo de 400 células mutantes para *rab5* es suficiente para contrarrestar los efectos de la competición celular y mantener el crecimiento del tumor.

4.- Las células normales en las cercanías de un tumor *rab5* activan su metabolismo y aumentan su tasa de proliferación. Éstas son reclutadas al tumor y contribuyen a su crecimiento.

5.- La proliferación extra y el crecimiento del tumor depende de la apoptosis y de la actividad de la vía JNK en las células del tumor.

6.- En los tumores *rab5* hay señalización de Dpp y Wg como consecuencia de la apoptosis y de la vía JNK.

7.- Proponemos un modelo en el que las células apoptóticas derivadas de la competición celular en el borde de los tumores *rab5* actúan como una fuente de mitógenos (Dpp y Wg) que inducen proliferación extra en células cercanas. Algunas de éstas se incorporan al tumor y adquieren su identidad, manteniendo un proceso dinámico que hemos denominado “modelo del agujero negro”. La competición celular juega en este contexto un papel promotor de tumores.

8.- La habilidad de Wingless para inducir proliferación extra en un modelo de células no-muertas depende de su capacidad para difundir desde el origen de la señal.

9.- La difusión de Wingless podría también estar implicada en la inducción de focos de células que expresan *wingless* ectópicamente en los tejidos hiperplásicos generados por las células no-muertas.

10.- Tenemos algunas vías y funciones celulares candidatas que podrían estar implicadas en el reconocimiento de células oncogénicas que pudiesen aparecer en tejidos en desarrollo.

Conclusions

1.- Oncogenic *rab5* mutant cells activate JNK and die through apoptosis if growing within a wild type context while they are viable in a homotypic environment. Cell competition plays a tumour suppressor role eliminating *rab5* mutant clones.

2.- A proliferative advantage conferred to *rab5* mutant clones facilitates clone merging and the formation of a protective microenvironment.

3.- A minimum size of about 400 *rab5* mutant cells is sufficient to overcome cell competition and sustain the growth of the tumour.

4.- Wild type cells at the vicinity of a *rab5* tumour activate their metabolism and increase their proliferation rate. These wild type cells are recruited to the tumour and contribute to its growth.

5.- The extra proliferation and tumour growth depend on apoptosis and JNK activity in tumour cells.

6.- There is Dpp and Wg signalling in *rab5* tumours downstream of apoptosis and JNK.

7.- We propose a model in which cell competition-derived apoptotic cells at the periphery of *rab5* tumours act as a source of mitogens (Dpp and Wg), inducing extra proliferation of cells at the vicinity. Some of these cells are recruited into the tumour and acquire its identity feeding a dynamic process that we have termed “the black hole model”. Cell competition plays in this context a tumour-promoter role.

8.- Wingless ability to induce extra proliferation in a model of undead cells depends on its capacity to spread form the source.

9.- Wingless spread could also be implicated in the induction of foci of ectopic *wingless*-expressing cells in the hyperplastic tissues generated by undead cells.

10.- We have several candidate pathways and cellular functions that may be involved in recognizing oncogenic cells that may appear in developing tissues.

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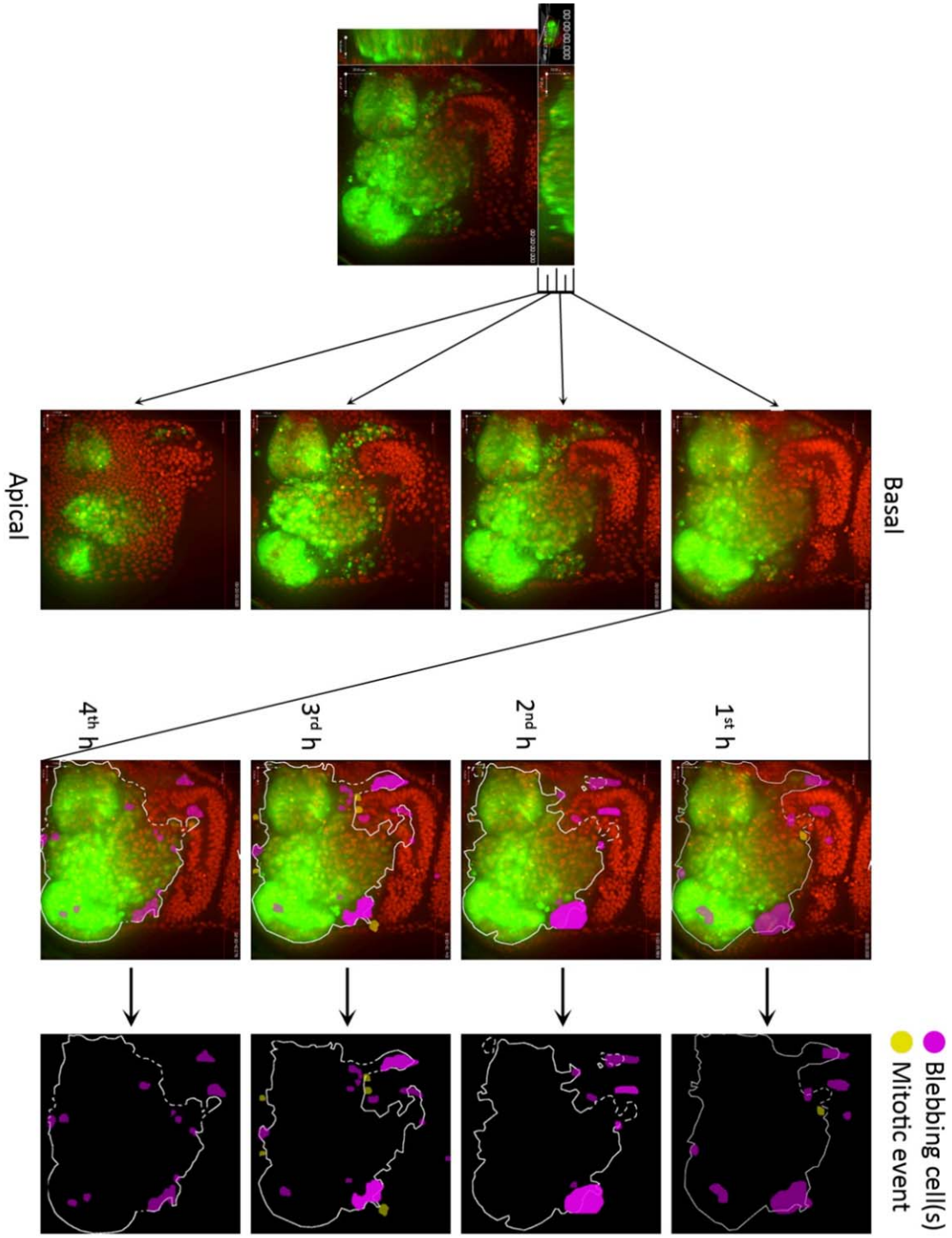
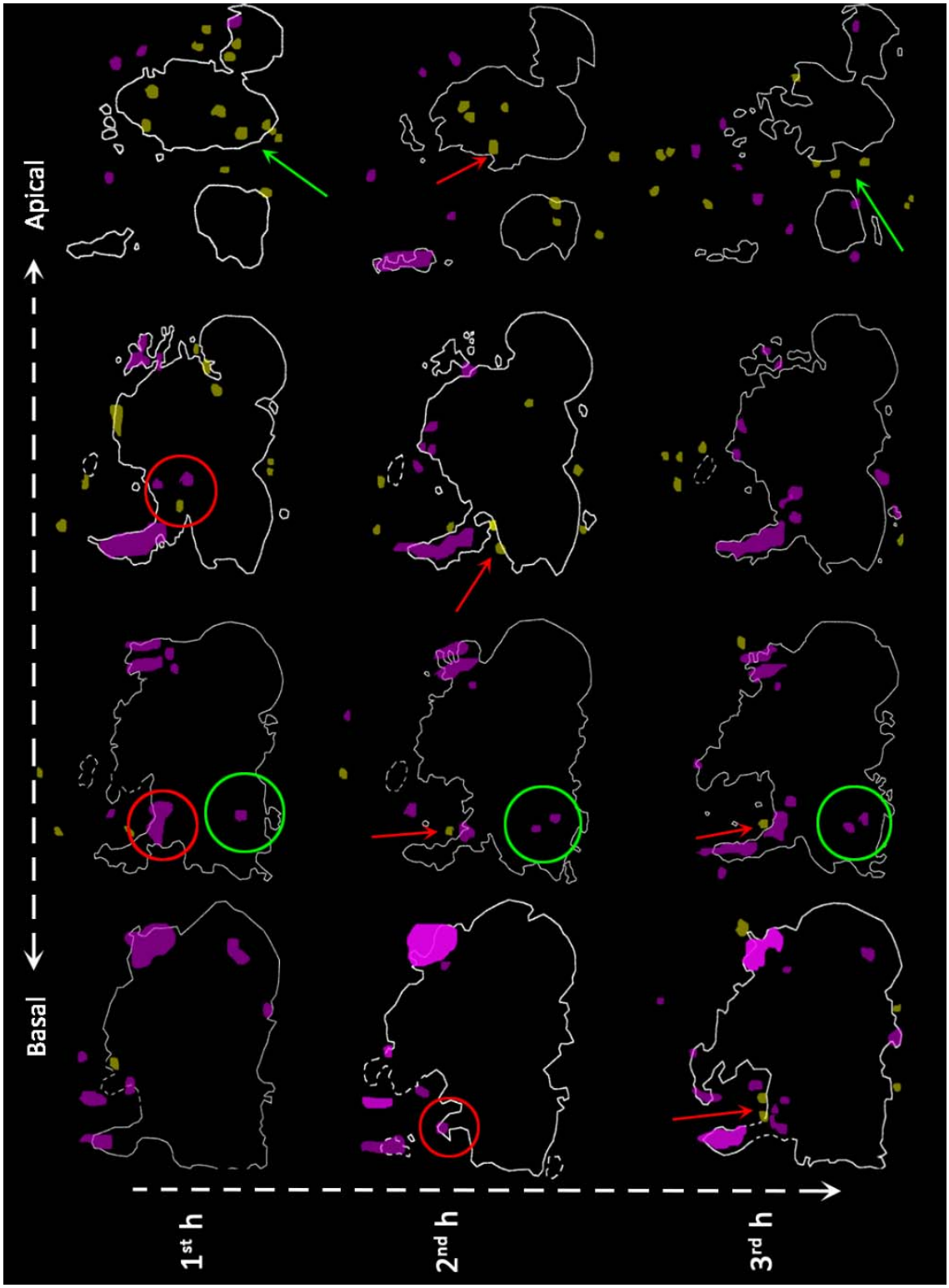
APPENDIX I: FIGURES, TABLE AND MOVIES

Appendix figure 1



Appendix figure 1. Scheme of the movie analysis of apoptotic and dividing events of a growing *sal^{EPv}>rab5^{KD}* tumour. Tumour cells are marked by the expression of *GFP* and all nuclei are marked by Histone-2-RFP. The original movie was taken for all the sections of the disc during 4h. To analyse the possible spatio-temporal relationship of the apoptotic and the dividing cells, we followed the steps we represent in this figure: a first apicobasal subdivision of the movie into four merged 4-h movies was followed by the separate analysis of each resulting movie into four 1-h movies. The resulting 16 movies were then analysed and a scheme was made to record all events happening in each movie in single images (apoptotic cells shown in purple and dividing cells in yellow).

Appendix figure 2



Appendix figure 2. In depth analysis of a movie of a growing *sal^{EPv}>rab5^{KD}* tumour. We have gathered together the resulting schematic view of the analysis of each individual movie. We did not further take into account the last hour of the movies due to the lack of dividing cells and the generalized apoptotic events, which were signs of lack of nutrients in the serum.

Appendix table 1

Gene	Biological process	Stock reference number
18wheeler	Humoral response, cell adhesion	Bloomington - 30498
5HT2	Serotonin receptor	Bloomington - 31882
anterior open	Positive regulation of apoptosis, trafficking	Bloomington - 26759
argos	Positive regulation of apoptosis, morphogenesis	Bloomington - 28383
ATP-synβ	Proton-exporting ATP synthase activity	Bloomington - 27712
bruchpilot	Synapse regulation, calcium channel	Bloomington - 25891
cactus	Antifungal humoral response	Bloomington - 31713
cad86C	Homophylic cell adhesion	Viena - 5097
calsyntenin1	Calcium-dependent homophylic cell adhesion	Bloomington - 25839
ced-12	Actin filament organization, cell migration	Bloomington - 28556
CG10738	Negative regulation of cell size, positive regulation of cell proliferation	Bloomington - 28580
CG14050	(Unknown)	Bloomington - 27073
Cyp4g15	Cytochrome P450 - electron carrier activity	Bloomington - 28077
dachsous	Planar polarity, heterophylic cell adhesion, proliferation	Bloomington - 28008
dap160	Negative regulation of Notch signalling	Bloomington - 25879
fasciclin1	Neural cell recognition, calcium-independent homophylic cell adhesion	Viena - 101779
flower	Calcium channel activity, positive regulation of apoptosis, cell competition	Bloomington - 27323
gr21a	Detection and respone to carbon dioxide, transmembrane receptor	Bloomington - 31598
gr59e	Perception of taste	Bloomington - 25815
Hdac3	Hiostone deacetylase, chromatin silencing, negative regulator of apoptosis	Bloomington - 34778
inflated	Integrin α-chain - cell adhesion	Bloomington - 27544
jaguar	Actin binding, intracellular transport	Bloomington - 28064
jra	Wound healing, JNK pathway transcription factor	Bloomington - 31595
kayak	Wound healing, JNK pathway transcription factor	Bloomington - 33379
kin of irre	Cell adhesion, embryo and muscle developent	Viena - 109585
ktl	Potassium channel	Bloomington - 25848
myoblast city	Organelle organization, tissue morphogenesis	Bloomington - 32355
neuralized1	Phosphatidylinositol phosphate binding, response to chemicals, positive regulator of apoptosis	Bloomington - 26023
obp44a	Sensory perception of chemical stimulus	Bloomington - 28794
pdcd4	Positive regulation of stem cell differentiation	Bloomington - 35712
peanut	Positive regulator of apoptosis	Bloomington - 27712
PGRP-LC	Peptidoglycan recognition protein, immune system (IMD)	Bloomington - 33383
phr	DNA repair	Bloomington - 27676
pointed	Transmembrane receptor, apoptosis regulator, hemocyte differentiation	Bloomington - 31936
prickle	Blockage of stimuli response, regulation of cell growth	Bloomington - 32413
pten	Actin cytoskeleton organizer, innate immune system, response to nutrient levels	Bloomington - 25841

relish	Innate immune system response to virus and gram positive bacteria, trancription factor	Bloomington - 33661
roundabout	Receptor activity, cell adhesion, cell motility	Bloomington - 31287
scab	Focal adhesion, migration, response to stress	Bloomington - 27545
septin1	Positive regulator of apoptosis, cytokinesis	Bloomington - 27709
septin2	Positive regulator of apoptosis, cytokinesis, regulation of cell cycle	Bloomington - 28004
spaguetti squash	Myosin filament assembly, morphogenesis	Bloomington - 7917
spätzle	Humoral immune response	Bloomington - 34699
src64B	Columnar/cuboidal epithelial cell differentition	Bloomington - 30517
starry night	Cell-cell adhesion, planar polarity	Bloomington - 26022
sticks and stones	Heterophylic cell adhesion	Viena - 109442
tollo	Innate immune response, receptor activity	Bloomington - 28519
αPS4	Cell recognition and cell adhesion	Bloomington - 28535
β3GalTII	Protein glycosylation	Bloomington - 31562

Appendix Table 1. Interference-RNAs used to screen for upstream regulators of cell competition. Here we show the list of candidate genes examined to date in the assay. The name of gene in *Drosophila*, a short summary of its functions and the stock reference are given.

Appendix movies 1 and 2 can be found in the CD accompanying this book. Their legends:

Appendix Movie 1. *Sal^{EPv} rab5^{KD}* tumour growing during 4h of recording. 40x magnification of a cultured disc centred in the ventral/wing region. The dorsal tip/notum region is not recorded. The view of the disc has been turned to better observe the events. The dorsal region is oriented to the top-right of the movie while the ventral region is located at the bottom-left of the view. The *Sal^{EPv}* domain is marked in green by the expression of *GFP*. All nuclei are marked by the expression of *Histone2-RFP*, in red. Wild type (*GFP$^-$*) cells located at the periphery of the domain acquire *GFP* expression during the course of the movie. The disc was dissected and cultured 72h after temperature shift (*rab5^{KD}* expression).

Appendix Movie 2. *Sal^{EPv} rab5^{KD}* tumour growing during 4h of recording (case II). 40x magnification of a cultured disc centred in the ventral/wing region. The dorsal tip/notum region is not recorded. The dorsal region is oriented to the top of the movie while the ventral region is located at the bottom of the view. Orthogonal sections of the disc are shown: a YZ section to the left of the disc; a XZ section to the top. The exact position of the sections shown here is outlined in the disc inset at the top-left of the image. The *Sal^{EPv}* domain is marked in green by the expression of *GFP*. All nuclei are marked by the expression of *Histone2-RFP*, in red. Wild type (*GFP$^-$*) cells located at the periphery of the domain acquire *GFP* expression during the course of the movie. The disc was dissected and cultured 72h after temperature shift (*rab5^{KD}* expression).

APPENDIX II: PUBLISHED MATERIAL

The work in this thesis has produced the following articles:

· Cell competition, apoptosis and tumour development. (2015) Ginés Morata and **Luna Ballesteros-Arias**. *International Journal of Developmental Biology*. **59**(1-3): 79-86.

· Developmental Biology. Death to the losers. (2014) Ginés Morata and **Luna Ballesteros-Arias**. *Science*. **346**(6214): 1181-2.

· Cell competition may function either as tumour-suppressing or as tumour-stimulating factor in *Drosophila*. (2014) Luna Ballesteros-Arias, Verónica Saavedra and Ginés Morata. *Oncogene*. **33**(35): 4377-84. Epub 2013.